June 2021

FORMATION, CHARACTERIZATION, AND UTILIZATION OF MULTILAYER NANOEMULSIONS IN FOODS

Jorge L. Muriel Mundo
University of Massachusetts Amherst

FORMATION, CHARACTERIZATION, AND UTILIZATION OF MULTILAYER NANOEMULSIONS IN FOODS

Jorge L. Muriel Mundo

FORMATION, CHARACTERIZATION, AND UTILIZATION OF MULTILAYER NANOEMULSIONS IN FOODS

A Dissertation Presented

By

Jorge Luis Muriel Mundo

Submitted to the Graduate School of the

University of Massachusetts Amherst in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 2021

The Department of Food Science

FORMATION, CHARACTERIZATION, AND UTILIZATION OF MULTILAYER NANOEMULSIONS IN FOODS

A Dissertation Presented

By

Jorge L. Muriel Mundo

Approved as to style and content by:

_____

David Julian McClements, Chair

_____

Jiakai Lu, Member

_____

Zhenhua Liu, Member (External)

_____

Lynne A. McLandsborough,

Food Science Department Head

DEDICATION

To my beautiful family, specially to my wonderful sister Chris Angelie Muriel, who have always demonstrated a beautiful resilience character against adversities.

ACKNOWLEDGEMENTS

I would love to take this opportunity to acknowledge a group of a couple of people who made all of this possible, without them, it is most likely that I would not had be able of accomplishing this doctoral journey, the way I did:

First and foremost, my greatest gratitude's go to the most amazing Advisor anyone could ever have Dr. David Julian McClements. Without him (Dr. McClements), I would not have been able of succeeding, on this long and challenging Graduate Life journey. Indeed, McClements humbleness and passion for research, inspired me and many others into becoming better scientists, but most importantly better human beings. McClements it is the most amicable but simultaneously the most intelligent person that I ever had the chance to personally met. However, even that his 8th ball pool-table game skills are not as high as his (h-index), Julian regardless will always be on my heart, and in my most precious memories from my arduous but beautiful times, here at UMass-Amherst. Hopefully, someday I could become half of the person that he is already today (April 1st, 2021).

Secondly, I would love to thanks Jean Alamed, she indeed is the crucial-key factor in our research-group, for every single aspect to succeed. Indeed, without her (Jean), not even Dr. McClements and/or Dr. Decker research-groups would be that successful as they both are today-day. Her patience, smile, and her charisma made my most stressful days, less painfully.

Thirdly, I would love to thanks to Mr. Louis Colaroutolo (hopefully a Dr. – someday), a person who became from an amanuensis (here your GRE word for the day), from my study partner, to a roommate, to a vacation trip partner to finally to one of my personal closest friends in less than 2 years. Interestingly, all of this would not has been possible, without the help of High Horse bar and Louis favorite bar = Stackers, located for your best

convenience at the North Pleasant Street here at UMass-Amherst. Thanks for everything Louis!!!!

Lastly, but never last Dr. David Johnson, a person that I considered my very first mentor in life, in sciences and even for professional matters. Moreover, I considered him one of my closest friends since I came to the USA (to dedicate my passion for Food Science research). His continuous feedbacks and mentorship have helped to become a better professional. He is the main reason that I chose UMass-Amherst to be my school and ultimately my home (during my Ph.D. years). When I first met David Johnson at our Kraft-Heinz 2015 summer internship, I quickly understood what it was needed to be done, in order to success in life. Thank you so much for everything my friend Dave!!

I would also, would love to take this opportunity to acknowledge my prestigious Puerto Rican family from here at Amherst, that without a doubt, I would not be here today without their emotional support and gracious times that we spent all together and we will remember for our entire lives. I would like to recognize (without any preferential order) to: Jessica Caballero Feliciano, Javier Mercado, Gabriela Ruiz Babilonia, Joann Hernandez, Isabella Camara, Jorge A. Rivera Cruz, Natasha Rivera, Marcos A. Amarberlt, Manuel "Picu" Diaz, Felix Salas, Jenny Muniz, Aurora Cotto, Pedro Matos Llavona, Jose Diaz, Michael Lu-Diaz and Emmanuel Rivera Iglesias for being part of what I considered; my Chosen Family. LOS AMO CON MI VIDA!!!!!!!!!!

ABSTRACT

FORMATION, CHARACTERIZATION, AND UTILIZATION OF MULTILAYER NANOEMULSIONS IN FOODS

MAY 2021

JORGE L. MURIEL MUNDO, B.S., Universidad Ana G. Mendez (UAGM), Carolina, P.R.

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Distinguished Professor David Julian McClements

Multilayer coatings have been proposed as a promising nanotechnology for improving the performance of emulsion-based products in numerous research fields. In foods, these multilayer coatings can be used to improve the encapsulation and protection of bioactive ingredients in delivery systems during storage and passage through the gastrointestinal tract (*1, 2*). Therefore, there is strong interest in understanding the formation, properties, and performance of these novel coatings. Multilayer coatings are formed by layer-by-layer electrostatic deposition of oppositely charged biopolymers, such as proteins and polysaccharides. A better understanding of the formation and properties of biopolymer multilayer coatings could lead to novel foods with improved performance. The purpose of this research was to improve our understanding of the fabrication and behavior of multilayer nanoemulsions suitable for application in the food industry.

First, the interaction of anionic ɣ-poly-glutamic acid (PGA) and cationic ε-poly-L-lysine (PLL) in solution was examined so as to better understand their behavior at interfaces. Electrostatic complexes were formed with a 1:4 mass ratio of polyanion-to-polycation at saturation (pH 7.4). The surface potential and aggregation stability of the complexes was highly dependent on solution pH (2 to 12), which was attributed to alterations in the electrical characteristics of the two polyelectrolytes. In particular, insoluble complexes were formed under pH conditions where there was a strong electrostatic attraction between the two polyelectrolytes, whereas soluble complexes were formed when there was only a weak attraction. The addition of salt (≥ 20 mM NaCl) promoted aggregation of the complexes, presumably due to screening of the electrostatic interactions between them. Conversely, temperature (25 to 90 ^{o}C) did not have a major impact on the stability of the complexes. These results may be useful for the design of effective oral delivery systems for bioactive agents in foods and other products.

Secondly, the same biopolymers were used to form multilayer coatings around the lipid droplets in oil-in-water nanoemulsions using a sequential layer-by-layer electrostatic deposition approach. Cationic poly-L-lysine (PLL) and anionic poly-glutamic acid (PGA) were used as a pair of oppositely charged polypeptides (pH 4.0). First, a primary emulsion (10% w/w soybean oil-in-water emulsion) was formed consisting of small lipid droplets (d32 = 500 μm) coated by a natural surfactant (0.05% w/w quillaja saponin). Then, cationic PLL was deposited onto the surfaces of the anionic saponin-coated droplets. Lastly, anionic PGA was deposited onto the surfaces of the cationic PLL-saponin-coated droplets. We then assessed the ability of the coatings to protect the lipid droplets from aggregation when the pH (2.0-9.0), ionic strength (0 to 350 mM), or temperature (30-90°C) were altered. The properties of the primary, secondary, and tertiary emulsions were monitored by measuring the mean particle diameter (d32), electrical characteristics (ζ-potential), and

microstructure of the lipid droplets. The electrical characteristics of the droplets could be modulated by controlling the number and type of layers used. The primary emulsion had the best resistance to varying environmental conditions, while the secondary emulsion had the worst, suggesting electrostatic deposition should only be used to obtain specific functionalities. Interestingly, PLL detached from the surfaces of the secondary emulsions at high salt concentrations due to electrostatic screening, which improved their salt stability. This phenomenon may be useful for some food applications, e.g., having cationic droplets during food storage, but anionic ones inside the human body.

Thirdly, multilayer coatings were formed from saponins, polypeptides, and polysaccharides using medium chain triglyceride (MCT) lipid droplets as templates (pH 4.0). First, an emulsion containing negatively charged lipid droplets was created using quillaja saponin (QS) as an anionic emulsifier. Second, these anionic droplets were coated with a cationic polypeptide (poly-L-lysine, PLL) to form positively-charged droplets. Finally, these cationic droplets were coated with a negatively-charged polysaccharide, either pectin (PE) or κ-carrageenan (KC), to form anionic droplets. Overall, the 1-layer emulsions had the best resistance to salt, pH, and heat, indicating that quillaja saponins were effective emulsifiers. The 2-layer emulsions had better pH-stability than the 3-layer emulsions, which tended to strongly aggregate under acidic conditions. Conversely, the 3-layer emulsions had better salt-stability than the 2-layer emulsions, which tended to aggregate strongly even at low salt levels (50-100 mM NaCl). All the emulsions were relatively stable to heating (90^{o}C, 30 min).

Fourth, the kinetics of β-carotene degradation in multilayer nanoemulsions was measured. Primary emulsions were formed containing anionic quillaja saponin-coated MCT oil droplets loaded with β-carotene. Secondary emulsions were then formed by depositing cationic polypeptide poly-l-lysine (PLL) onto these anionic droplets. Tertiary emulsions

were then formed by depositing anionic poly-glutamic acid (PGA), pectin (PE) or κ-carrageenan (KC) onto these cationic droplets. All the multilayer emulsions were prepared at pH 4.0 to ensure the biopolymers had opposite charges. The kinetics of β-carotene degradation in the different emulsions were then measured when they were incubated at 55°C. In addition, changes in the particle size and ζ-potential of the emulsions were measured using light scattering methods. The chemical stability of the encapsulated carotenoid was highly dependent on the nature the nature of the coating used. The secondary emulsions, which had cationic PLL as an external layer, gave the best protection against color fading, with a final yellowness (b*) of 82% after two weeks. Conversely, the tertiary emulsions, which had anionic polysaccharides or polypeptides as an external layer, gave the worst protection. For instance, when KC was used as the external layer the final yellowness was only 32% after two weeks. These results show that the stability of carotenoids can be improved by controlling the properties of multilayer coatings around oil droplets.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Pages

LIST OF FIGURES

Figure Page

LIST OF COMMON ABBREVATIONS

QS: Quillaja Saponin “Q-Naturale”

PLL or ε-PLL: Epsylon-Poly-L-Lysine

PGA or ɣPGA: Polyglutamic Acid

LbL: Layer-by-Layer

Kc or KC: κ-carrageenan

PE: Pectin

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

The cosmetic, pharmaceutical and food industry sectors, are just one of the few industries that heavily rely on the use of emulsions and emulsifiers to encapsulate, protect and deliver bioactive compounds or lipophilic drugs to their customers. Specifically, the most common emulsifiers used in the food industry-sector are amphiphilic proteins, polysaccharides, phospholipids and small molecule surfactants. However, there are still some current limitations to the functional properties that can be achieved using existing food emulsifiers and the conventional method of creating emulsions, for example, limited stability to pH, salt, heating, dehydration, freezing and chilling (*3*). The physicochemical properties, stability, and functional attributes of emulsions can be modulated by engineering their interfacial properties using layer-by-layer deposition methods to produce multilayers emulsions (*4-6*). Multilayer O/W emulsions consist of oil droplets dispersed within an aqueous continuous phase, with each oil droplet being coated by a laminated interfacial coating, which usually consists of emulsifier and biopolymer molecules(*7*). On **(fig 1.1.)**, Multilayer emulsions can be fabricated using a multistep procedure: (1) *primary emulsion*: an oil and aqueous phase are homogenized together in the presence of an electrically charged hydrophilic emulsifier; (2) *secondary emulsion*; an oppositely charged polyelectrolyte is added to coat the droplets; and (3) *multilayer emulsions*: sequential polyelectrolyte adsorption steps can be carried out to add additional layers(*8*).

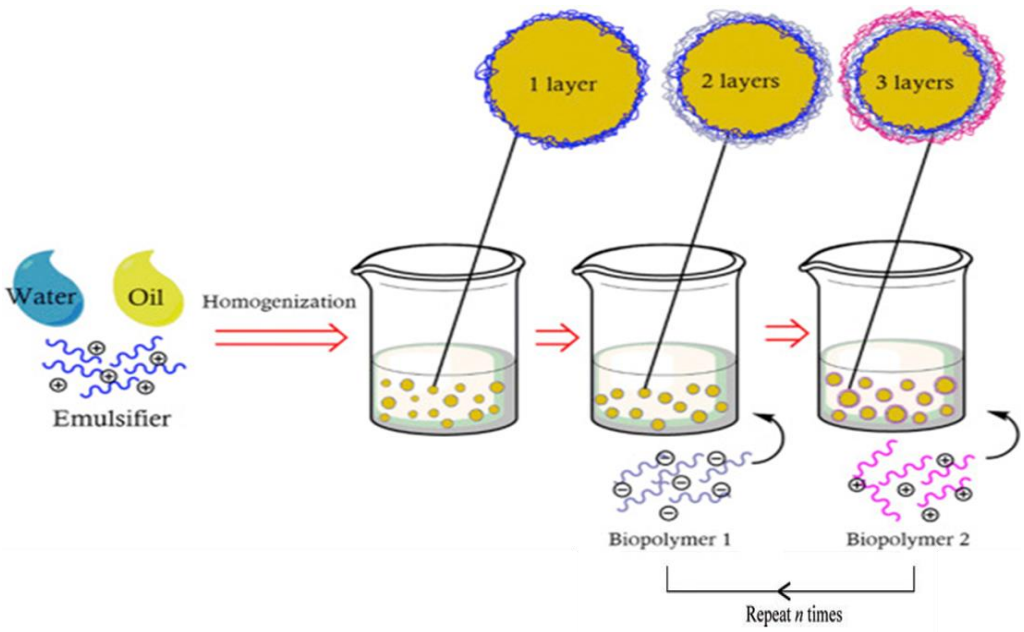


Figure 1.1. Layer-by-Layer (LbL) assembling via *Saturation Method*.

For this reason, in my projects (*chapter 3-5*), I emphasized the use of laminating coating to engineer emulsion surfaces-membranes droplets **(fig 1.1.)**, thereby, modulating lipid droplets physiochemical functionalities on aqueous solutions. Which consisted of stabilizing labile hydrophobic bioactive compounds (such as soybean oil, β-carotene, etc.) by using nanotechnology approaches that involve forming multilayer laminated coatings around nanosized oil droplets using natural biopolymers, such as polypeptides and dietary fibers. For this reason, my research has led to several scientific publications (3) as first author (2 with others submitted or in preparation), and several others as co-author (14). My first published paper involved the preparation and characterization of electrostatic complexes formed from two oppositely charged polypeptides (*9*) (chapter 2). My second published paper was to use these polypeptides to form multilayers around the oil droplets in soybean oil-in-water emulsions and improve their stability and functionality

(*10*)(chapter 3). Then, my third published paper was about to find food-grade alternatives to the polypeptides, such as dietary fibers (*11*) (chapter 4). Lastly, I applied different ways to retard β-Carotene degradation, by using multilayer(s) system technology approaches and additionally, I proposed the studies of β-Carotene degradation within the incorporation of Ascorbic Acid (strong food-grade hydrophilic antioxidant) (pre-liminary experiments data can be found in the *Appendix-supplemental material* section) on this written thesis. Surprisingly, the results of this research should lead to the development of innovative approaches to stabilize vitamins, nutraceuticals, and healthy oils in emulsion-based foods using commercially viable approaches.

1.2 Literature Review

1.2.1: Multilayer Particle Characteristics

The structural and physicochemical characteristics of Multilayers (layer-by-layer, LbL) systems can be controlled, as can the characteristics of the laminating coatings, for example, composition, thickness, charge, permeability, environmental responsiveness, and chemical reactivity. Knowledge of the physicochemical principles governing the formation and properties of these multilayer interfaces is important to establish the optimum conditions required for producing multilayer emulsions with desirable properties. Under certain circumstances, emulsions containing oil droplets surrounded by multilayer interfaces have been found to have better stability to environmental stresses than conventional oil-in-water emulsions with single-layer interfaces(*12, 13*).

1.2.1.1 Composition and Structure

Multilayer coatings can be assembled from food-grade emulsifiers and biopolymers that are charged, like ionic surfactants, phospholipids, proteins, or polysaccharides. Interestingly, numerous of food-grade emulsifiers and polyelectrolytes can be used to assemble the laminated coatings around oil droplets, including electrically charged surfactants, phospholipids, proteins, polysaccharides, and particulates. Food-grade emulsifiers and biopolymers with different molecular characteristics (e.g., electrical charge, molecular weight, conformation, hydrophobicity, and thermal stability) and functional properties (e.g., solubility, viscosity, gelation and surface activity) can be used, which gives one a great deal of scope in designing the functional performances of laminated coatings. For example, the functional performance of coatings can be controlled by:

- Changing the nature of *emulsifier* used to prepare the initial lipid droplets.
- Changing the nature of the *polyelectrolytes* used to form the individual layers within the laminated coatings.
- Changing the total *number* of electrostatic deposition steps (and therefore layers) used to prepare the laminated coatings.
- Changing the *order* that the various polyelectrolytes (P) are deposited onto the lipid droplet surfaces (e.g., E-P_1/P_2/P_3 versus E-P_3/P_2/P_1)
- Controlling the properties of the *solutions* used during the preparation of the laminated coatings (e.g., pH, ionic strength, and solvent quality).
- Cross-linking one or more of the polyelectrolytes layers in the laminated coatings, for example, physically, chemically, or enzymatically.

Lastly, one or more of this approaches could be ideally combined during the assembling process to produce more synergistic and versatile multilayers delivery systems(*3, 14-21*).

1.2.1.2 Dietary Fibers

Dietary Fibers (Polysaccharides) are usually isolated from plant or microbial sources and may vary considerably in their molecular and physicochemical properties, such as molecular weight, branching, flexibility, charge, polarity and digestibility(*22, 23*). Dietary fibers are also known to have an impact on the behavior of lipids within the gastrointestinal tract and can therefore be used to modulate the response of humans to ingested lipids(*24*). Therefore, dietary fibers may influence lipid digestion through a variety of mechanisms: (i) they may bind to species that play a critical role in digestion, such as bile salts, phospholipids, enzymes or calcium(*25*), (ii) they may increase the viscosity of the intestinal phase, and thereby alter mass transport processes(*26, 27*); (iii) they may form protective coatings around lipid droplets thereby inhibiting lipase access(*28-30*) (iv) they may promote lipid droplet aggregation thereby changing the amount of lipid surface exposed to lipase(*31, 32*); (v) they may inactive digestive enzymes (*24, 33, 34*); (vi) they may alter the microbial population within the large intestine (*35*). The ability of dietary fibers to impact lipid digestion through these and other mechanisms ultimately depends on their molecular and physicochemical properties.

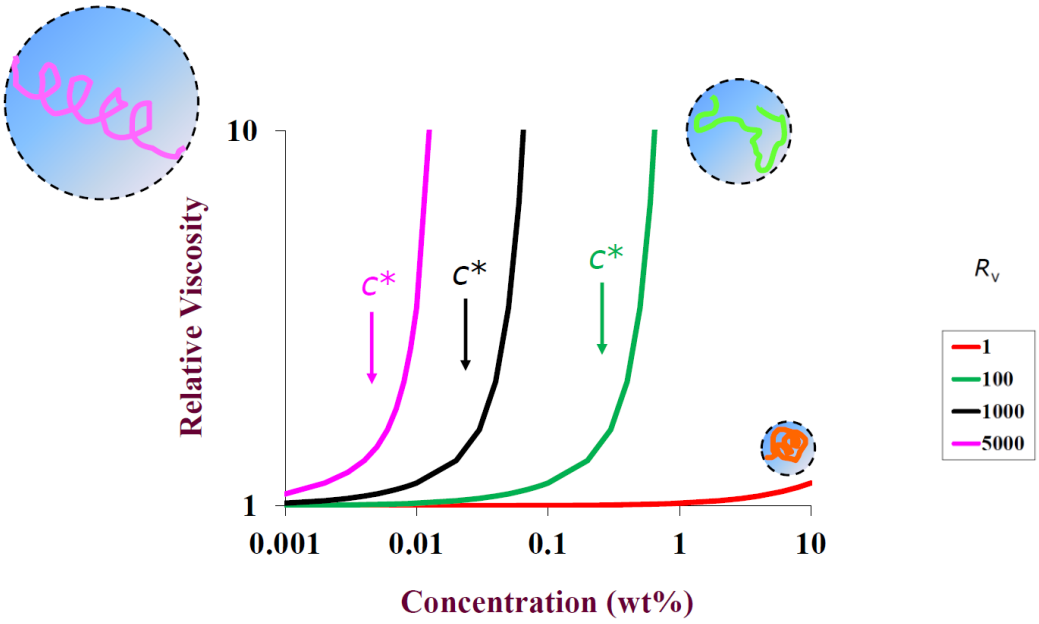


Figure 1.2. Dietary Fibers; Conformations-Concentrations vs Relative Viscosity are often used in food matrixes as *Thickening* agents, conveniently to reduce gravitaional separation rate on emulsions system.

There are a large number of different polysaccharides commercially available and recognize as GRAS, to form multilayers(*22*). Recently, natural polysaccharides have shown to be very useful for drug entrapment and sustained release of drug. The natural polymers used as carrier materials in the encapsulation technology have the great advantage of being nontoxic, biocompatible, and biodegradable(*36*). As we stated earlier, that the laminating coating used to assemble a multilayer system could be influenced by many characteristics such as *conformation* of the biopolymer utilized during layer-by-layer (LbL) electrostatic deposition fabrication process. For this reason, we did a couple of experiments (*chapter 4-5*) where the external tertiary layer was ruled either by linear or branch dietary fiber conformations; κ-carrageenan and Pectin (as tertiary external layers). These two dietary

fibers, were excellent representatives of either linear (κ-carrageenan) and/or branched (Pectin) polysaccharides conformations. We are now currently intending to replace and/or incorporate different polysaccharides in our multilayer system (here detailed), just to see how this will influence in terms of multilayers stability and/or in the modulation of calorie intake during lipid digestion.

Pectin

Pectin with an overall molecular weight of around 110 to 150 kDa, is a branched polysaccharide with a linear backbone containing many ionizable carboxylic acid groups ($pK_a \approx 3.5$), as well as numerous "hairy regions" consisting neutral side chains extending from the backbone(*37*). The linear backbone is mainly comprised of α(1-4)-d-galacturonic acid residues that may be partly esterified with methyl groups. Pectin is not digested by gastric or small intestinal enzymes but is easily degraded by pectinases produced by the colonic microflora. LM pectin is more tolerant of pH variations and calcium levels, which could make it more suitable for colonic delivery systems formation. The charge characteristic of pectin can be selected to control lipid digestion and bioactive release, *e.g.,* decreasing DE reduced lipid digestion and bioactive bioavailability.

κ-carrageenan

Carrageenans are natural anionic compounds that are normally extracted from red seaweeds(*38*). These polymers are linear chains of D-galactopyranosyl units joined with alternating (1 → 3)-a-D- and (1 → 4)-b-D-glycosidic linkages, with most sugar units having one or two sulphate half-ester groups. These sulphate groups are responsible for the

negative charge of the polymer, as they are always ionized at the pH values present in foods. There are three major types of carrageenan, kappa (k), iota (i), and lambda (l)-carrageenans(*39*). However, κ-carrageenan is one of the most common forms of carrageenan's used in foods, and it is characterized by having D-galactose-4-sulphate, 3,6-anhydro-D-galactose-2-sulphate as a building block, and has a double-helix conformation(*38, 40*). The popularity of this ingredient in the food industry is due to the ability of its linear helical portions to associate to form a three-dimensional gel in the presence of appropriate cations.

Alginate

Alginate is a block copolymer composed of 1-4-linked residues of β-D-mannuronic acid (M) and α-L-guluronic (G) acid, with an overall molecular weight around 60 kDa to 700 kDa. Alginate ability to form gel under electrostatic cross-linking interaction between multivalent cations (typically Ca2+) has been also used to for chitosan–alginate multilayer beads cross-linked with polyphosphate by developing stables, non-toxic, interpolymer complex of ionic-cross-linked chitosan–alginate–tripolyphosphate (TPP) beads with improved drug release properties(*41*) Calcium alginate hydrogels shrink at low pH (gastric environment) due to the loss of negative charge on the alginate molecules when the carboxyl groups become protonated (-COOH, pKa ≈ 3.5). Moreover, Alginate can be further cross-linked or mixed with other polymers such as neutral gums, chitosan or pectin to strengthen the structure and restrict the pore of the gel network. Although the self-assembled multilayer film of alginate with several cationic polymers has been increasingly been reported(*14, 42-45*).

Chitosan

The biopolymer, the N-deacetylated product of the polysaccharide Chitin; Chitosan is one of the few examples of positively charged polysaccharides, the presence of ionizable amino groups (-NH3+, pKa ≈ 6.3) is the origin of the cationic charge of this biopolymer(*46*). It has a linear structure composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units, with an overall average molecular weight around 3.8 to 20 kDa(*47*). One of the many reasons that it has been incorporated to delivery system is due to its great capacity of encapsulating, protecting and releasing lipophilic bioactive agents due to their mucoadhesive properties during oral-digestions(*24*). Ultimately Chitosan is gaining importance in the pharmaceutical field owing to its unique polymeric cationic character, good biocompatibility, non-toxicity and biodegradability. Chitosan has been proposed as a useful excipient for either sustained release of water-soluble drugs and for enhancing the bioavailability of poorly water-soluble compounds(*41*).

1.2.1.3 Proteins

Proteins are also commonly used food biopolymer for the fabrication of multilayer systems. It should be noted that many proteins and/or polypeptides may exhibit anti-oxidant properties, which makes them particularly suitable for the protection, encapsulation and/or laminating interfacial coating of lipophilic bioactive agents that are susceptible to oxidation, such as carotenoids and ω-3 oils(*48-53*). Their incorporation as interfacial coating layers during the development of multilayer systems are mostly intended for lipid oxidation reduction rate purposes(*49*). However, considerations of fabrication conditions involved during these layer-by-layer assembling's such (e.g., pH, ionic strength, temperature) where polypeptides or proteins are involved, needs to be always monitored

and controlled. Remembering, that proteins are sensitive to physical and chemical conditions around the protein molecule: pH, ionic strength and temperature will affect protein conformation, same goes to polypeptides. Binding and interaction with other molecules (substrate, cofactors, other proteins) will also alter protein conformation. If the conformation is altered too drastically, the protein molecule may lose function, it breaks and it becomes denatured. Some proteins have more stable conformations if they are stabilized by disulfide bonds.

Here I will be emphasizing mostly on proteins that has been already successfully electrostatically deposited onto opposite charged surfaces droplets in the past, or proteins-polysaccharides that we will be intending to incorporate in our multilayer system model promptly (*later discussed*).

Whey Protein Isolated (WPI)

Whey proteins have an isoelectric point around pH 5, and so are positively charged below this value and negatively charged above it. They can therefore be used to assemble structures based on electrostatic attraction, such as coacervates and multilayers. In addition, whey proteins are surface-active and can be used to form oil-in-water emulsions(*49*). However, whey protein-coated lipid droplets are particularly sensitive to pH, ionic strength, and temperature(*52, 54, 55*). Whey protein actually contains numerous different globular protein fractions, with the most abundant being β-lactoglobulin (β-Lg), α-lactoalbumin (α-La), bovine serum albumin (BSA), and immunoglobulins with molecular weights ranging from 14 kDa to 1,000 kDa. WPI has been used in the past for lipid oxidation

protection, as an emulsifier under acidic conditions (pI ≈ 5) where whey protein stills exhibit an strong cationic (ζ-Potential) (+ mV) surface charge, ideally, to electrostatic repulse transition metal such as Fe^{2+} and Cu^{2+} away from the ω-3 poly-unsaturated fatty acids (e.g., salmon oil) on the O/W lipid droplets(*48*).

Casein

Casein belongs to the heterogeneous group of phosphoproteins precipitated from raw skimmed milk[63].Caseins are amphiphilic molecules that have good surface-activity and can therefore be used as emulsifiers to stabilize lipid droplets. They have an isoelectric point around pH 4.6, and are positive at lower pH and negative at higher pH. There is a few types of caseins, used on laboratory settings; (1) α(s1)-casein has molecular weight (MW) 23,000. The chain is build from 199 amino acids with 17 proline residues. It has two hydrophobic regions, containing all the proline residues, separated by a polar region, which contains all but one of eight phosphate groups and is highly charged. Molecular diameter of α(s1)-casein is 9 nm. In the other hand, α(s2)-casein: MW 24,000; 207 residues, 10 prolines. Negative charges are concentrated near N-terminus and positive charges near C-terminus. Both caseins can be precipitated at very low levels of calcium. Molecular weight of β-casein is 24,000. It has 209 residues and 35 prolines. N-terminal region is highly charged, hydrophilic and Cterminal region is a hydrophobic. This amphiphilic protein acts like a detergent molecule. It is less sensitive to calcium precipitation. Its molecular diameter is 7.5 nm. MW of κ-casein is 19,000, 169 residues with 20 prolines. Casein molecules, casein micelles or casein-coated oil droplets tend to aggregate strongly around their isoelectric point due to the reduction in electrostatic repulsion at this pH[64]. Moreover, caseins possess a number of favorable characteristics suitable for the development of hydrogel

biomaterials, such as high hydrophilicity, good biocompatibility particularly in oral delivery applications, lack of toxicity and availability of reactive sites for chemical modification. In aqueous solution single protein behaves as flexible, disordered, polyelectrolyte-like molecule[65], therefore, it is easily integrated into polyelectrolyte films[1, 66]. Lastly, casein has ability to bind calcium ions and therefore, it can be used in biotechnology and in biomedical applications. Thus, materials covered with casein containing films can be also applied in diary industry for the prevention of calcium deposit formation[66].

Lactoferrin

Lactoferrin (LF) is an iron-binding glycoprotein that contains about 680 amino acid residues and has a molecular weight of about 80 kDa[67]. The surface of the lactoferrin molecule has several regions containing clusters of cationic groups, giving it a high isoelectric point (pI ≈ 8.5), and making it positively charged over a wide range of pH values encountered in food systems[68]. The presence of positively charged lactoferrin-coated droplets provides an opportunity to modify the interfacial properties by depositing negatively charged substances onto the cationic droplet surfaces[69]. At pH 7.0, the LF is positively charged, increasing the relevance of (LF) for layer-by-layer system oral-digestive routes applications . However, in previous studies it was shown that the conjugation of (LF) with a polyphenol decreased the isoelectric point of the protein to around pH 4.5[70-71]. Therefore, at pH 7.0, the LF is positively charged, whereas the LF–polyphenol is negatively charged, and so they have been used due to the forming of complexes through electrostatic attraction to protect lipophilic bioactive agents such as β-Carotene, in the past[71].

β-Lactoglobulin

β-Lactoglobulin (BLG) is the major whey protein of cow and sheep's milk (~3 g/l), and is also present in many other mammalian species; a notable exception being humans. (BLG) is positively charged at pH's below its isoelectric point (pI ≈ 5.2) and negatively charged at pH values above this value[13]. (BLG) is also the dominant globular protein found in whey[72], and its molecular and physicochemical properties have been well documented[73]. (BLG) has a molecular weight of 18.4 kDa, and a thermal denaturation temperature (T_m) of ≈ 70 °C, at neutral pH[74]. Biopolymer nanoparticles based on (BLG) could be formed by heating them in the presence of either anionic or cationic polysaccharides, such as beet pectin and chitosan.[72] Then, in another study, β-lactoglobulin was used as a primary cationic emulsifier and pectin as an anionic biopolymer for the production of the secondary emulsions [75]. Later, by mixing the droplets and biopolymer at a pH where they initially had similar charges (pH 7) and then adjusting the pH to values where they had opposite charges led to the formation of *more stable* secondary emulsions than directly mixing the droplets and biopolymer at pH values where they had opposite charges (pH 3)[76]. Ultimately, is demonstrated that when (BLG) biopolymer is utilized as a coating layer onto emulsion droplets, is strictly dependent on pH, ionic strength, and temperature ideal conditions, for a higher performance[8].

1.2.2: Food Multilayers Fabrication Methods

There is an existing variety of fabrication methods that facilitates the assembling of a particular food-grade multilayer system. However, each of every single method here included, are enthalpy ruled by a differential electrostatic charge gradient, leading to a strong interaction between polyelectrolytes and surfaces. They all need the presence of free

opposite charged polyelectrolytes under specific conditions, for a multilayer layer assembling to take place. Existing considerations for bridging and depletion flocculation are present in each described method. For the sake of clarity, bridging flocculation occurs when a polyelectrolyte adsorbs to the surface of more than one droplet and links them together. In the other hand, depletion flocculation occurs when the free polyelectrolyte concentration in the continuous phase generates an attractive osmotic force that is strong enough to overcome the various repulsive forces[3].

(1) Saturation Method

It is possible to add just enough polyelectrolyte to completely coat all of the particles present in the system, so that there is little free polyelectrolyte remaining in the aqueous phase **(fig 1.1)**. The saturation concentration for a particular system has to be determined empirically (for example using ζ potential measurements). One has to be careful to add enough polyelectrolyte to prevent bridging flocculation, but not too much as to cause depletion flocculation (see later). The origin of this osmotic force is the exclusion of polyelectrolyte molecules from a narrow region surrounding the droplet surfaces. This method can be repeated a number of times, and thereby, increasing the thickness of the multilayer system (changes on particle molecular weight, relative viscosity, density, and visual appearance on aqueous samples, are just a few parameters to consider).

(2) Centrifugation Method

In this method a solution that contains more than enough polyelectrolyte than required to completely saturate the particles present is added to a colloidal suspension. Any

excess non-adsorbed polyelectrolyte molecules are then removed by centrifuging the colloidal suspension, collecting the particles, and resuspending them in an appropriate buffer solution. This procedure can be repeated several times to ensure that all of the free polyelectrolytes has been removed, before the next polyelectrolyte material solution is added. The main problem with this method is that it can promote particle aggregation during the centrifugation step because the particles are forced into very close proximity. Thereby, temporarily reducing any possible electrostatic repulsion existing among particles, during the addition of the consequent polyelectrolyte coating material. Presumably, leading to the entrapment of more than one single lipid droplet under a same single layer polymer coating material.

(3) Filtration Method

In this method a solution that contains more than enough polyelectrolyte than required to completely saturate the particles present is also added to the colloidal suspension. However, in this case the excess non adsorbed polyelectrolyte molecules are removed by membrane filtration of the colloidal suspension. A filter is used that allows the polyelectrolyte molecules to pass through, but not the colloidal particles. The colloidal suspension is put under pressure, which forces the aqueous phase containing the excess polyelectrolyte through the filter. At the same time, a buffer solution can be added to the colloidal suspension to keep the overall volume of the system constant. In this way, the colloidal particles are never forced into close proximity, which reduces the amount of particle aggregation that occurs in the system. Another advantage of this method is that it is

not necessary to have a density difference between the particles and the surrounding liquid[3].

For the three methods mentioned above one must carefully control the system composition and preparation conditions, to form stable multilayer colloidal particles. For example, it is important to ensure that there is sufficient polyelectrolyte present to cover all the surfaces (droplets membranes) present, that there is not too much free polyelectrolyte present to promote depletion flocculation; and, that the polyelectrolyte adsorbs more rapidly than when particle–particle collisions occur. These processes depend on the size and concentration of the polyelectrolyte molecules and colloidal particles present, as well as on the solution conditions (e.g., pH, ionic strength, dielectric constant, temperature, and stirring[3, 16].

Other Methods

I should also mention that in some systems, it is indeed possible to mix the colloidal particles and polyelectrolyte molecules together at a pH where they do not have opposite charges, and then adjust the pH to a value where they do have opposite charges so as to promote polyelectrolyte adsorption. This procedure can be carried out when the charge on the polyelectrolyte or colloidal particle can be varied by altering the pH, e.g., the charge on a protein goes from positive to negative when the pH is adjusted from below to above its isoelectric point. The advantage of this method is that the polyelectrolytes are evenly distributed throughout the continuous phase before adsorption occurs, which should ensure uniform and rapid polyelectrolyte adsorption and help prevent droplet aggregation[3].

1.2.2.1: Multilayer Properties

Multilayer's composition, thickness, charge, packing and environmental responsiveness can be tailored by manipulating the laminating coating material surrounding the oil droplets, thereby, having a responsible premeditated effects on the physicochemical and functional properties in general. Strategically, the properties of laminated coatings **(fig 1.3)** can also be engineered to alter other physicochemical or functional attributes of multilayer emulsions[3-4, 40, 77]:

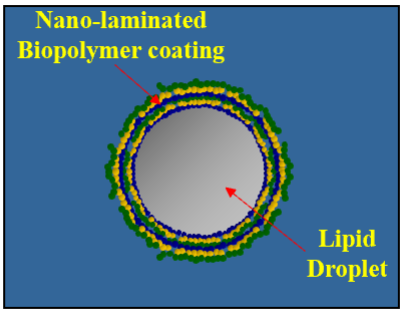


Figure 1.3. Multilayer System Design

- Coating properties can be altered to improve the physical stability of emulsions to environmental stresses, such as pH, ionic strength, thermal processing, chilling, freezing, dehydration, and mechanical agitation. Improved physical stability can often be achieved by manipulating the thickness and charge on the polyelectrolyte coatings so as to increase the steric and electrostatic repulsion between particles[16].

- Coatings can be designed to retard the chemical degradation of labile encapsulated

components. For example, the oxidation of ω-3 fatty acids and carotenoids can be reduced by limiting the interactions between pro-oxidant transition metal ions in the aqueous phase and unsaturated lipids within the droplets. This can be achieved by controlling interfacial charge, thickness, and permeability. For example, cationic interfaces will repel cationic transition metals from the droplet surfaces, whereas thick dense interfaces will inhibit the diffusion of transition metals to the lipid droplet surfaces[16].

- Coating properties can be designed to control the release of encapsulated active agents due to the ability to manipulate thickness, permeability, and environmental responsiveness of the interfacial layer. For example, a polyelectrolyte layer could be designed so that it remains attached to the oil droplet surfaces under one set of conditions (e.g., specific pH, salt, or temperature range), but becomes detached under another set of conditions[16].

In summary, the major advantage of multilayers technology is that interfacial coating with specific physicochemical properties (e.g., thickness, charge, permeability, composition, and digestibility) **(fig 1.3.)** it can be designed to achieve a particular functional performances (e.g., antioxidant properties, stability to environmental stresses, controlled digestibility, and targeted release profiles)[16]. Therefore, the electrical properties of the first layer of a multilayer emulsion are determined by the emulsifier, and they can therefore be controlled by selecting different types of emulsifier[78-79]. Hence, consequent multilayer coatings can be assembled from food-grade charged emulsifiers and biopolymers, like ionic surfactants, phospholipids, proteins, or polysaccharides, using a sequential electrostatic deposition

method[23, 80-81]. The selection of the most appropriate combination of coating materials is critical to forming a stable system with the desired functional attribute[77]. Moreover, it has been seen that when a variation of protein-polysaccharide coating material is interchangeable used around an emulsion droplet, versatile adaptations could be immediately conferred[20, 46, 75-76].

1.2.2.2: Multilayer Applications

Perhaps until this moment, you may be wondering how-why *multilayers system*s are useful? Surprisingly, multilayer emulsions have several of very useful applications in the food industry. For example, a thick highly charged multilayer interfaces may be useful in protecting droplets against aggregation or in preventing lipid oxidation. On the other hand, multilayer interfaces that change their properties in a controlled fashion in response to some environmental condition could be used for controlled or triggered release of active ingredients[3]. However, these trigger responses are mostly ruled by the exogenous conditions surrounding the laminating coating polymers-materials protecting the lipid droplet. Hence, the laminating chosen to assemble a multilayer system, plays a crucial role in response to these environmental conditions.

Stability improvement against environmental stresses

Food emulsions experience a variety of different environmental stresses during their manufacture, storage, transport, and domestic utilization. Which can include pH extremes drifts, high ionic strengths, thermal processing, freeze–thaw cycling, drying and mechanical-manual agitation. Many of the emulsifiers currently available for utilization

within the food industry can only provide a limited protection against some of these environmental stresses[3]:

Ionic Strength

The presence of salts (e.g., monovalent salts) can alter interfacial and emulsion properties through a variety of physicochemical mechanisms, including changing the amount of polyelectrolyte adsorbed, altering the structure of the interfacial layer, or modulating the strength and range of the various colloidal interactions between the droplets[3]. Specifically, sodium chloride have the ability of screening electrostatic interactions between opposite charged polyelectrolytes in a complex or in a layer-by-layer (LbL) system[18]. Unfortunately, the concentration and/or presence of monovalent salt ions, tends to vary considerably depending on the nature of the food, the purity of the functional ingredients, and the hardness of the water used to prepare the emulsion[16]. For this reason, often the application of just a single emulsifier, is not enough to overcome all these monovalent salt variations and other hurdles. Moreover, some studies have shown that emulsions containing droplets coated by interfacial layers formed from an anionic surfactant and cationic chitosan have better stability to high salt concentrations than those coated by anionic surfactants alone[21, 82]. For this reason, multilayer assembling has increased their relevance and use, on food industries, where in cases electrically charged food-grade biopolymers can be used to form multilayer coatings around the lipid droplets in oil-in-water emulsions using a sequential layer-by-layer electrostatic deposition approach.

pH

The aqueous phase pH may vary during the production, storage or utilization of the food product. For example, in coffee creamers the pH of the aqueous solution surrounding the protein-coated oil droplets changes from around pH 7 in the original product to around pH 5 when the creamer is poured into hot coffee[83]. It is often important to ensure that that the oil droplets do not aggregate when they are exposed to variations in pH. The influence of pH on the tendency of oil droplets to aggregate is normally determined by how the various repulsive forces generated by the interfacial layer vary with pH, e.g., electrostatic and steric repulsion. Multilayer interfaces are normally produced using weak polyelectrolytes, and so their thickness, structure and electrical characteristics are strongly dependent on solution pH. By manipulating the type of polyelectrolytes used to prepare multilayer emulsions it is therefore possible to control the influence of pH on droplet aggregation[3].

Heating (thermal processing)

Many food emulsions undergo some form of thermal processing during their production, storage or utilization, e.g., pasteurization, sterilization or cooking[16]. Therefore, in occasions breaking down due to droplet flocculation or coalescence[3]. Many emulsifiers are unsuitable for creating droplets that are resistant to thermal processing because they undergo changes in their ability to prevent droplet aggregation with temperature. Interestingly, multilayer emulsions containing droplets coated by an anionic surfactant and a cationic polyelectrolyte have been shown to be stable to thermal processing from 30 to 90 °C, e.g., SDS–chitosan[82], lecithin–chitosan[21] and SDS–gelatin[22]. For a number of these systems, the stability of the secondary emulsions to heating was better than that of the

primary emulsion, which was partly attributed to the increased steric repulsion between the droplets. Multilayer emulsions containing droplets coated by protein–polysaccharide complexes have also been shown to have better stability to thermal processing than those stabilized by proteins alone[3].

Chilling and freezing

Many oil-in-water emulsions become physically unstable when they are chilled and/or frozen, and rapidly break down after reheating. It is therefore important to have technologies improve the stability of food emulsions to chilling, freezing and thawing. Understanding, that chilling and freezing is a very common process often used in the food industry sector for the controlling of microorganisms growth and either for food preparation purposes, such; e.g., dairy products, desserts, sauces, and ice cream products[3]. A variety of different physicochemical processes may occur when a food emulsion is cooled, including fat crystallization, ice formation, freeze-concentration, interfacial phase transitions, and biopolymer conformational changes. When oil-in-water emulsions are cooled to temperatures where the fat phase becomes partially crystallized (but the aqueous phase remains liquid) they become susceptible to a phenomenon known as *partial coalescence*[16]. At present there is still a relatively poor understanding of the relative importance of these various mechanisms of emulsion instability for particular systems. Nevertheless, previous studies have indicated that some multilayers can indeed improve the stability of oil-in-water emulsions to chilling and freezing processes, which suggests it is able to retard or prevent some of these instability mechanisms[21, 82].

Lipid Oxidation

One of the most fascinating roles of O/W emulsions, it is indeed the ability of incorporating poly-unsaturated fatty acids (such as ω-3 fatty acids), due to their potential health benefits e.g., there is evidence that they decrease the risk of coronary heart disease, immune response disorders, and mental illnesses[84]. Nevertheless, incorporation of these oils into food products is problematic because they are highly susceptible to oxidative degradation resulting in rancid off-flavors, which has greatly limited their more widespread usage[3, 57, 85]. Some of the instabilities that have been seen primary emulsions, have been attributed to low strong electrostatic repulsion effects, a thinner interfacial membrane (low steric repulsions) and/or an anionic behavior that tends to attract positively charged Fe^{2+} ions through membrane absorption[3, 62]. Any of these previously mentioned hurdles, can be overcome by the addition of an additional or multiple opposite charged layers, around the primary emulsifier layer. In occasions, when a secondary-tertiary external coating layer is electrostatically deposited onto a O/W emulsion surface, a noticeable change in electrostatic repulsion intensities, a thicker membrane and a cationic behavior can be immediately conferred [9, 19, 62, 75, 86]. Therefore, providing versatile abilities to electrostatically and/or sterically repulse transition metals (e.g., $Cu2^{+}$ or $Fe2^{+}$) and/or free radicals further away from the poly-unsaturated lipid droplets (where ω-3 fatty acids, is already entrapped)[56-58, 84, 87]. Fascinatedly, multilayers technology implementation could potentially impact the lipid oxidation emulsions field, like no other food-grade colloidal system[4, 23, 78, 88-92].

Dehydration

Oil-in-water emulsions are often converted into a powdered form in the food industry to increase their shelf life, reduce transport costs and/or facilitate their utilization. This microencapsulation process is normally carried out by evaporating the majority of water from the emulsion using a suitable dehydration method, such as spray drying or freeze drying[93]. Often single-primary emulsifiers cannot survive similar spray drying heating conditions. Moreover, lipid phase content (%) can be limited when only a single emulsifier is used, thereby, potentially increasing transport and production costs. In the other hand, multilayers technology may help in producing higher quality emulsion droplets and additionally, increasing final lipid phase content (%) and facilitating dehydration to take place[3].

Controlled release/Triggered release

Multilayers technologies have the ability to encapsulate, stabilize and deliver a variety of functional food components e.g., flavors, bioactive lipids, enzymes, peptides, antimicrobials and antioxidants[3]. Specifically, due to multilayers capacity to carry oil-soluble, water-soluble, and amphiphilic functional agents in different regions inside of a multilayer architecture. The multilayer technique has the major advantage that wall characteristics, such as thickness, charge and permeability, can be finely tuned by careful selection of polyelectrolytes and preparation conditions. Interestingly, the release time (during oral digestion) will depends on the nature of the first adsorbed layer and the number of polyelectrolyte layers composing the shell wall[94]. The bioactive agent release target location for oral digestion purposes, should be premeditated during the multilayer electrostatic assembling-encapsulating step. An example, considerations of each isoelectric

point, pH responsiveness from every single layer utilized during the multilayer assembling process are crucial to hypothesis, where the bioactive agent is going to be released inside a human body[85, 95-96]. For this reason, *in-vitro* INFOGEST is an excellent method to simulate multilayers controlled release performances; by the presence of enzymes, mechanical agitations and change in pH during different gastrointestinal stages via oral digestion, plays a huge role for the coating engineering in the layer-by-layer (LbL) assembling process knowledge[70, 97-101].

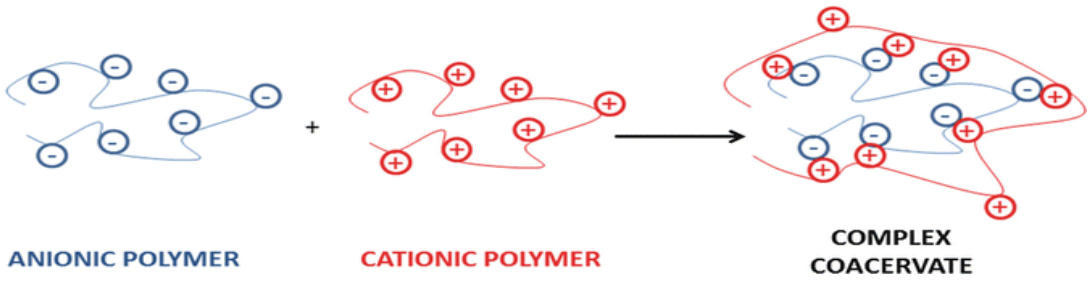
1.3 Conclusion

In summary, the major advantage of the multilayer technology is that interfacial coatings with specific physicochemical properties (e.g., thickness, charge, permeability, composition, and digestibility) can be designed to achieve a particular functional performances (e.g., antioxidant properties, stability to environmental stresses, controlled digestibility, and targeted release profiles). Nevertheless, they are more expensive to fabricate than conventional emulsions, and have to be prepared carefully to avoid droplet flocculation[16]. However, these multilayered emulsions have better stability to environmental stresses than conventional emulsions under certain conditions. More research is still needed to establish, at a fundamental level, the factors that influence the preparation of stable multilayered emulsions with specific functional attributes, including emulsifier characteristics (e.g., sign and magnitude of droplet charge), polyelectrolyte characteristics (e.g., molecular weight, charge density and flexibility), and mixing conditions (e.g., ionic strength, pH, temperature, stirring, order of addition), and washing conditions (e.g., ionic strength, pH, temperature, stirring, filtration and centrifugation). In addition,

research needs to be carried out to establish where these multilayered emulsions can be practically used within the food industry[3].

CHAPTER 2. CHARACTERIZATION OF ELECTROSTATIC INTERACTIONS AND COMPLEX FORMATION OF ɣ-POLY-GLUTAMIC ACID (PGA) AND Ɛ-POLY-L-LYSINE (PLL) IN AQUEOUS SOLUTIONS.

Table of Content:



ɣ-PGA ε-PLL

2.1 Introduction.

Complex coacervation is a form of liquid-liquid phase separation that occurs when two polyelectrolytes associate with each other through electrostatic attraction [10-11, 79, 102-108]. Typically, the two polyelectrolytes have opposite net charges (one positive, one negative), however, it is possible for two polyelectrolytes with similar net charges (both positive or both negative) to form complexes, provided there are enough positive groups on one and negative groups on another [11]. Many different synthetic and natural polymers have been studied for their ability to form polyelectrolyte complexes through this phenomena, including food-grade proteins and polysaccharides [107, 109]. These complexes have been developed for applications in the food, cosmetics, paper, textiles, and pharmaceutical industries as delivery systems for flavors [109-110], vitamins [111], and drugs [107, 110]. For instance, active agents have been encapsulated to improve their environmental stability, to separate

them from incompatible ingredients, mask off-flavors, reduce gastric irritation, and provide prolonged or sustained release. A number of different fabrication methods have been developed to produce complex coacervates, involving both physicochemical and mechanical methods [111]. Microencapsulation by complex coacervation is being increasingly used in the food industry because of its high encapsulation efficiency and mild processing conditions [102]. Moreover, microcapsules produced by coacervation can be designed to be resistant to environmental stresses, as well as to create controlled- or triggered-release profiles based on changes in mechanical stresses, temperature, pH, or ionic strength [110]. Moreover, they can encapsulate and protect bioactive ingredients during storage and passage through the gastrointestinal tract [9-10].

After two attractive polyelectrolytes are mixed together, the dense liquid phase formed, which is relatively concentrated in both polyelectrolytes, is called the coacervate [11]. In the food industry, proteins and polysaccharides are often used to form coacervates, but they only form these electrostatic complexes over a narrow range of pH and ionic strength where the two biopolymers have appropriate electrical characteristics [12]. In particular, it is often difficult to form coacervates around neutral pH conditions because many proteins and polysaccharides have a strong negative charge, which leads to a strong electrostatic repulsion. This limitation can be overcome by using polyelectrolytes that have opposite charges at neutral pH.

In this study, we used anionic ɣ-poly-glutamic acid (ɣ-PGA) and cationic ε-poly-L-lysine (ε-PLL) as two oppositely charged peptides to form polyelectrolyte complexes. Previously, it has been reported that these two polyelectrolytes assemble into electrostatic complexes at pH 7.40, which consist of alternating layers of the two peptides held together by electrostatic attraction and hydrogen bond (β-sheet) formation [79]. PGA is an edible

anionic polymer with high water-solubility and good biodegradability that is produced by microbial fermentation (*Bacillus subtilis*). Studies have already shown that this polymer has a range of potential applications in food, pharmaceutical, and healthcare products [112]. As an example, the Japanese traditional food, ''natto'' (fermented soybeans), is a mixture of ɣ-PGA and fructan produced by Bacillus natto [112-113]. The anionic moieties (carboxylic acid groups) on ɣ-PGA have been reported to have a pKa value of around 4.86 [114], and so it should be negatively charged over a wide range of pH found in foods.

ε-PLL was chosen as an edible cationic polymer that also has good water-solubility and biodegradability [115]. The cationic moieties (primary amino groups) on ε-PLL have a pKa value around 10 [92, 116], which means this polyelectrolyte has a positive charge over a wide range of pH values. Several studies have shown that (ε-PLL) has potential applications as a functional ingredient in food products, particularly for controlling microbial growth because of its high antimicrobial activity, low toxicity, high water-solubility, and good thermal stability [46, 117-126]. The incorporation of ε-PLL into foods, however, can be difficult because of its bitter taste and tendency to form complexes with anionic ingredients, leading to turbidity, sedimentation, and loss of functionality. Studies have shown that when cationic ε-PLL interacts with anionic polysaccharides it can form electrostatic complexes that may be soluble (transparent) or insoluble (turbid) depending on solution composition [118-120]. Consequently, it may be possible to overcome some of the challenges associated with incorporating ε-PLL into foods by making judicious use of electrostatic complexation.

The purpose of the current study was to examine the factors that impact the formation and properties of electrostatic complexes of ε-PLL and ɣ-PGA. This information would facilitate the rational design of biopolymer-based delivery systems for nutraceuticals,

vitamins, and other bioactive agents intended for oral consumption. In general, the formation of polyelectrolyte complexes is impacted by many factors, including polymer properties (e.g., molar mass, conformation, charge density, functional groups, and hydrophobicity), polymer content (e.g., mixing ratio and total concentration) and solution conditions (e.g., pH, ionic strength, and temperature) [11, 103, 127].

Keywords: microencapsulation; complex coacervation; polylysine; polyglutamate; polyelectrolyte complexes

Polyelectrolyte:	**Ionic Behavior:**	**pI or pKa:**	**Application in Food(s):**	**Produced by Microbial Fermentation:**
ɣ-poly-glutamic acid (PGA)	Anionic	(carboxylic groups) have a pKa value around 4.86.	In a mixture of fermented soybeans or fermented subproducts.	Bacillus subtilis[112] or Bacillus licheniformis and/or Bacillus anthracis[112]
Epsilon-poly-L-lysine (ε-PLL)	Cationic	(amine groups) have a pKa value around 10.	As an antimicrobial growth agent.	Streptomyces albulus[128]

Table #2.1. Basic descriptions of ɣ-PGA and ε-PLL.

2.2 Materials and Methods

Materials

Epsilon-poly-L-lysine (MW_{AVG} = 2.5 to 3.8 kDa) (CAS No. 73548-20-60) and ɣ-poly-glutamic acid (MW ≥ 700 kDa) (CAS No. 25513-46-6) were purchased from Wilshire Technologies Inc. (Princeton, NJ, USA) and stored in dry ambient temperature conditions prior to use. All other chemicals were of analytical grade. Double distilled water from a water purification system (NANOpure Infinity, Barnstead International, Dubuque, IA) was used for the preparation of all solutions.

Preparation of Polyelectrolyte Solutions

An aqueous ε-PLL solution was prepared by dispersing powdered ε-poly-l-lysine into double distilled water to reach a final concentration of 0.5 w/v %. An aqueous ɣ-PGA solution was prepared by dispersing powdered ɣ-poly-glutamate into double distilled water to reach a final concentration of 2 w/v%. These solutions were then adjusted to pH 7.40 using NaOH or HCl solutions and then stirred for at least for 3 h at ambient temperature to ensure dissolution. Solutions were then stored overnight before being used. Before every experiment, the solutions were vortexed and mixed to ensure they were homogeneous.

Electrical charge and particle size characteristics

Microelectrophoresis and dynamic light scattering were used to determine the electrical characteristics and dimensions of the polyelectrolytes and their complexes. The ζ-potential of the particles in the polyelectrolyte solutions was measured using a commercial particle electrophoresis instrument (Zetasizer Nano-ZS, model ZEN3600, Malvern

Instruments, Worchester, U.K.). The same instrument was used to measure the dimensions of the particles based on dynamic light scattering. All measurements were conducted on at least two freshly prepared samples and repeated three times per sample. Samples were diluted using double distilled water adjusted to pH: 7.40 to avoid multiple scattering.

Isothermal Titration Calorimetry (ITC)

An isothermal titration calorimeter (VP-ITC, Microcal Inc., Northampton, MA) was used to measure the enthalpy changes associated with the polyelectrolyte interactions at 25.0°C. Twenty-nine 10 μL aliquots of ɣ-PG solution (2.0 w/v% pH 7.40) were injected sequentially into a 1480 μL titration cell initially containing either water (adjusted to pH 7.40) or 0.5 w/v% ε-PLL dissolved in water (adjusted to pH 7.40). As a control, twenty-nine 10 μL aliquots of water were injected into 0.5 w/v% ε-PLL dissolved in water (both previously adjusted to pH 7.40). Each injection lasted 12 s, and there were intervals of 360 s between injections.

Turbidity Measurements

Influence of polyelectrolyte ratio

The impact of polyelectrolyte ratio on the formation of large insoluble complexes was determined using turbidity measurements. Aliquots of 2% ɣ-PGA solution (0 to 2000 μL) were injected into a 0.5% ε-PLL solution (initially 7.5 mL), both at pH 7.40. Each sample was then mixed and vortexed to ensure it was homogeneous before the turbidity (τ_{550}) was measured at a wavelength of 550 nm using a UV-Visible spectrophotometer (Genesys 150, Thermo Fisher, Madison, WI 53711, USA) at ambient temperature (around

25°C). The samples and reference (distilled water) were contained in 1 cm pathlength optical cells.

Influence of salt

The turbidity (τ_{550}) of the ε-PLL and ε-PLL-ɣ-PGA solutions were measured in the absence or presence of salt using the same UV-visible spectrophotometer at ambient temperature. Samples containing salt were prepared by dispersing powdered sodium chloride into the polymer solutions, followed by stirring until the salt was completely dispersed throughout the solution. HCl or NaOH solutions were then used to adjust the system to pH 7.40. The resulting samples were then stirred and stored overnight at room temperature.

Influence of temperature

A number of samples were selected for thermal analysis based on the results from the turbidity measurements carried out at ambient temperature. The turbidity of these samples was then measured using a UV-visible spectrophotometer (Ultraspec 2000, Pharmacia Biotech) when they were heated from 25 to 90°C at 5°C/min. All these experiments were conducted with at least two freshly prepared samples.

Statistical analysis

All data were collected from at least two individual experiments. Each independent experiment was conducted on at least two replicates. The results were expressed as the

average ± standard errors (SE) of these combined values. Statistical analysis was conducted using Excel (Microsoft, Redmond, VA, USA).

2.2.1: Poly-L-Lysine

ε-Polylysine (ε-PLL) has a light yellow to milk-white powder appearance and is slightly bitter in taste whether in powder or liquid form and is used commercially as a food preservative in Japan, Korea and in imported items sold in the United States (food products containing (ε-PLL) are mainly found in Japan). The use of poly-l-lysine (ε-PLL) is common in food applications such as boiled rice, cooked vegetables, soups, noodles and sliced fish (sushi). Literature studies have reported an antimicrobial effect of ε-polylysine against yeast, fungi, Gram-positive and Gram-negative bacteria. Moreover, Polylysine (ε-PLL) have potential applications as a functional ingredient in food products, particularly for controlling microbial growth because of its high antimicrobial activity, low toxicity, high water-solubility, and good thermal stability [117-118, 120, 122-123, 125-126]. The production of ε-polylysine by natural fermentation was first described by researchers Shoji Shima and Heiichi Sakai in 1977[128]. Since the late 1980s, polylysine has been approved by the Japanese Ministry of Health, Labour and Welfare as a preservative in food. In January 2004, polylysine became generally recognized as safe (GRAS) certified in the United States.

2.2.1.1 Molecular and physicochemical characteristics

Chemical formula and structure

Polylysine refers to several types of lysine homopolymers, which may differ from each other in terms of stereochemistry and link position. The precursor amino acid lysine

contains two amino groups, one at the α-carbon and one at the ε-carbon. Either can be the location of polymerization, resulting in α-polylysine or ε-polylysine. Polylysine is a homopolypeptide belonging to the group of cationic polymers: at pH 7, polylysine contains a positively charged hydrophilic amino group. However, ε-Polylysine (ε-poly-L-lysine, EPL) is typically produced as a homopolypeptide of approximately 25–30 L-lysine residues *$(C_6H_{12}N_2O)n$* **(Figure 2.1.)**, thereby, production of polylysine by natural fermentation is only observed in strains of bacteria in the genus *Streptomyces*. *Streptomyces albulus* is most often used in scientific studies and is also used for the commercial production of (ε-poly-L-lysine) [129].

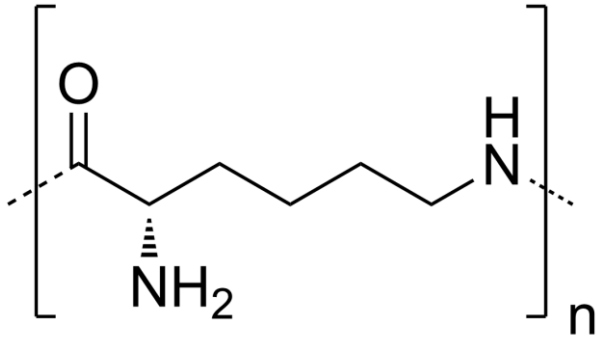


Figure 2.1. Chemical structure of ε-Poly-L-Lysine (ε-PPL)

Polymer (ε-PPL) properties

ε-Poly-L-Lysine (ε-PLL) has lysine monomer linked by α-carboxyl group and ε-amino group of lysine. The cationic moieties (primary amino groups) on (ε-PLL) have a pKa value around 10 [117]. Moreover, ε-PPL is cationic when the environmental pH is lower than its isoelectric point (pI ≈ 9.0), because of the presence of primary (-NH3 +) amine groups along its backbone[92]. Absorption of bioactive through oral digestion in humans, tends to be

affected by the hydrocolloid's molecular weight and/or density. Molecular weight of Epsilon-poly-L-lysine (ε-PPL) (MW_{AVG} = 2.5 to 3.8 kDa), used for the remainder of the experiments included on this thesis) (were acquired from Wilshire Technologies Inc, Princeton, NJ, USA). Hence, (ε-PPL) formula weight is 158.24132 g/mol when *($C_6H_{12}N_2O$) * HCl* is found in the chloride-powder state. The density of (ε-PPL) tends to vary among manufactures and still not very clear on literature yet. Interestingly, melting point from Epsilon-poly-L-lysine can be found anywhere around this high temperatures ranges either from 142.2 °C up to 172 °C, depending of course on manufacturer biopolymer extraction procedures. Moreover, also the boiling point temperature ranges tends to vary from manufactures and suppliers.

2.2.2.: Poly Glutamic Acid polypeptide

PGA is an edible anionic biopolymer with high water-solubility and good biodegradability that is produced by microbial fermentation (*Bacillus subtilis*). Studies have already shown that this polymer has a range of potential applications in food, pharmaceutical, and healthcare products [130]. As an example, the Japanese traditional food, ''natto'' (fermented soybeans), is a mixture of ɣ-PGA and fructan produced by Bacillus natto [113, 130]. In PGA, the amide linkages are formed between the α-amino group and the -carboxyl group in the polymer backbone.

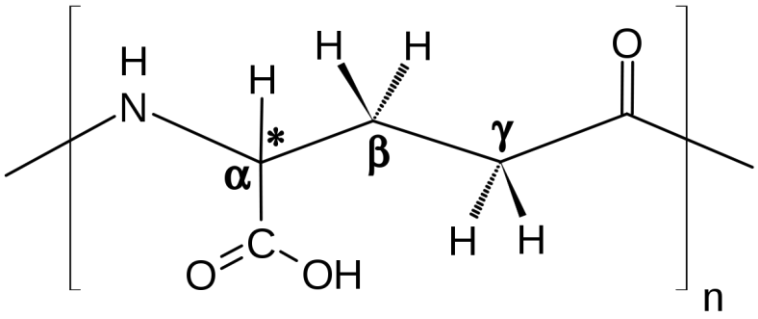


Figure 2.2. Chemical Structure of Polyglutamic Acid (PGA)

Molecular and physicochemical characteristics

Polymer (ɣ-PGA) properties

The anionic moieties (carboxylic acid groups) on ɣ-poly-glutamic acid (ɣ-PGA) have been reported to have a pK_a value of around 4.86 [114], and so it should be negatively charged over a wide range of pH found in foods[131]. Moreover, (ɣ-PGA) have a chemical formula of $(C_5H_7NO_3)n$ is detailed to have (1.409 g/cm^3) on density, with a molecular weight (MW = 387.34200 Da) on average. However, some variability can be found from different manufacturers and/or suppliers. In the other hand, (ɣ-PGA) melting point still not very clear on the literature. However, it has been found that in a temperature of 333.8ºC at 760 mmHg (ɣ-PGA) tends to boil (boiling point). Moreover, with a defined flash point around 155.7 º C temperature range.

Table #2.2. Production of PGA in Solid State Fermentation (SSF)[112].

STRAIN	KEY NUTRIENTS REQUIREMENTS
B. LICHENIFORMIS NCIM 2324	Soybean meal, citric acid, glutamic acid (NH4)2SO4, glycerol, L-glutamine, c-ketoglutaric acid
B. SUBTILIS ME714	Sodium glutamate, urea trisodium citrate, starch
B. SUBTILIS CCTCC202048	Soybean cake powder, wheat bran, glutamic acid, citric acid, NH_4NO_3
B. SUBTILIS CCTCC202048	Swine manure, soybean cake, wheat bran, glutamic acid, citric acid
B. SUBTILIS CCTCC202048	Dairy manure, wheat bran, soybean cake, glutamic acid.

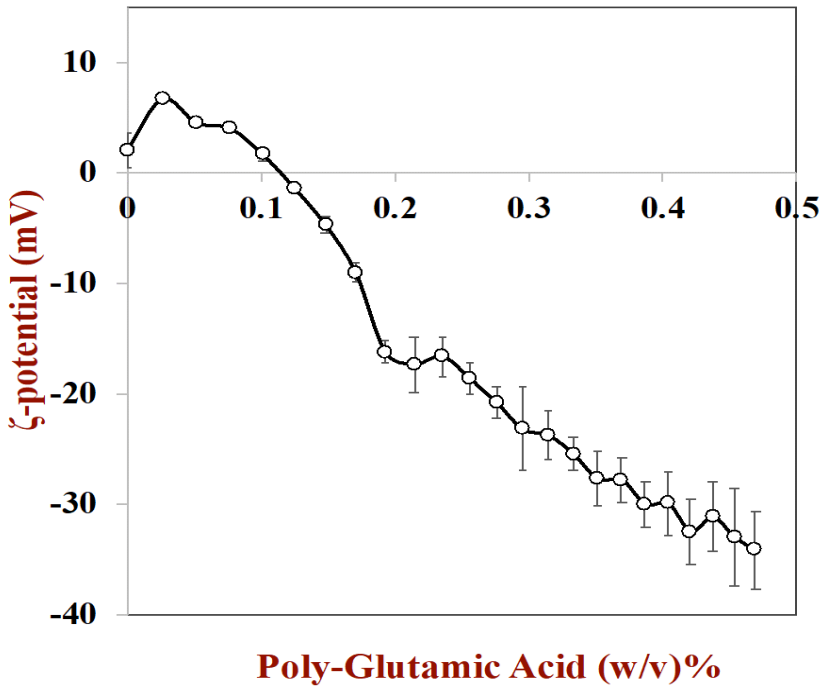


Figure 2.3 Surface potential (ζ-potential) of 0.5% w/w poly-L-lysine solutions when increasing amounts of poly-glutamic acid were added (pH 7.4).

2.2.2.1: Complex Formation

Surface potential analysis

The surface potential (ζ-potential) of the polyelectrolytes and their complexes was measured at pH 7.40 using microelectrophoresis **(Figure 2.3)**. In these experiments, aliquots of 2% ɣ-PGA solution were injected sequentially into a 0.5% ε-PLL solution to vary the mass ratio of the two polyelectrolytes. In the absence of ɣ-PGA, the measured surface potential was relatively small and positive (ζ ≈ +2 mV), which can be attributed to the presence of some $-NH_3^+$ groups along the backbone of the ε-poly-l-lysine structure. The relatively low magnitude of the charge suggests that the linear charge density of this

polymer was relatively low. This may have been due to association of counter-ions, such as Cl^-, with the amino groups. Indeed, polylysine is supplied in a hydrochloride form. As the ɣ-PGA level was increased, the ζ-potential of the mixed system became less positive and then more negative, with a point of zero charge around 0.12 %. This effect suggests that the anionic ɣ-PGA molecules bound to the cationic ε-PLL molecules and formed a complex. Presumably, the carboxyl groups on the ɣ-PGA chains bound to amino groups on the ε-PLL chains. Initially, this led to charge neutralization and a reduction in the net positive charge of the complexes. Eventually, all the amino groups on the ε-PLL were occupied and so adding any more ɣ-PGA caused an increase in negative charge. This may have been because there was a change in the stoichiometry of the complexes or because of the presence of free (non-complexed) ɣ-PGA in the aqueous phase.

Turbidity analysis

In general, two polyelectrolytes can form either soluble or insoluble electrostatic complexes depending on the nature of the system (Kizilay et al., 2011). Turbidity measurements were therefore used to detect the presence of any large insoluble complexes in the mixtures. Initially, the pure ε-PLL solution was optically transparent, indicating that the polylysine was fully soluble under these conditions. The addition of increasing levels of ɣ-PGA to the ε-PLL solution led to a progressive increase in the turbidity until a maximum value was reached around 0.125 % polyglutamate, after which the turbidity progressively decreased until the solutions became clear at high polyglutamate levels.

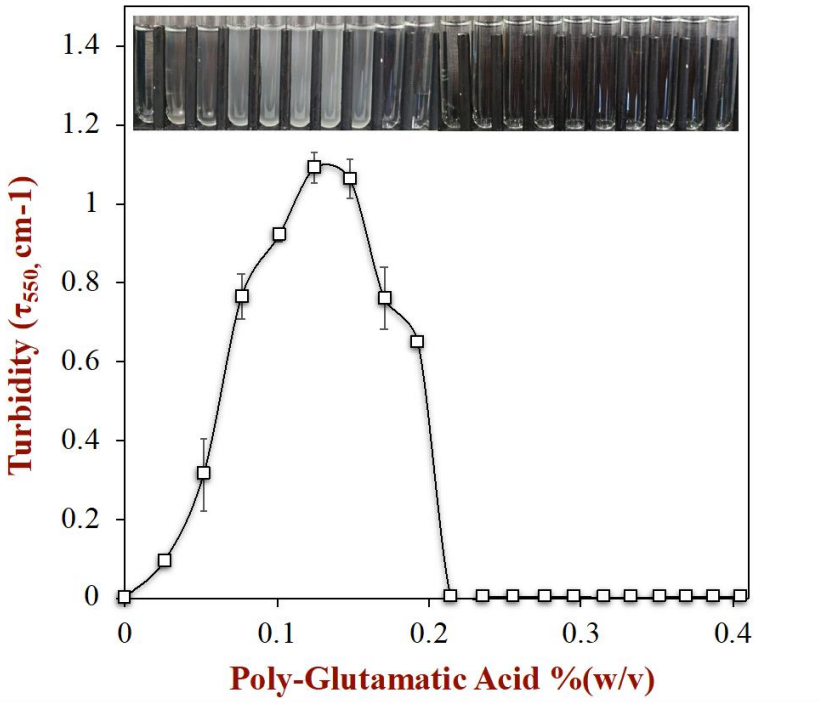


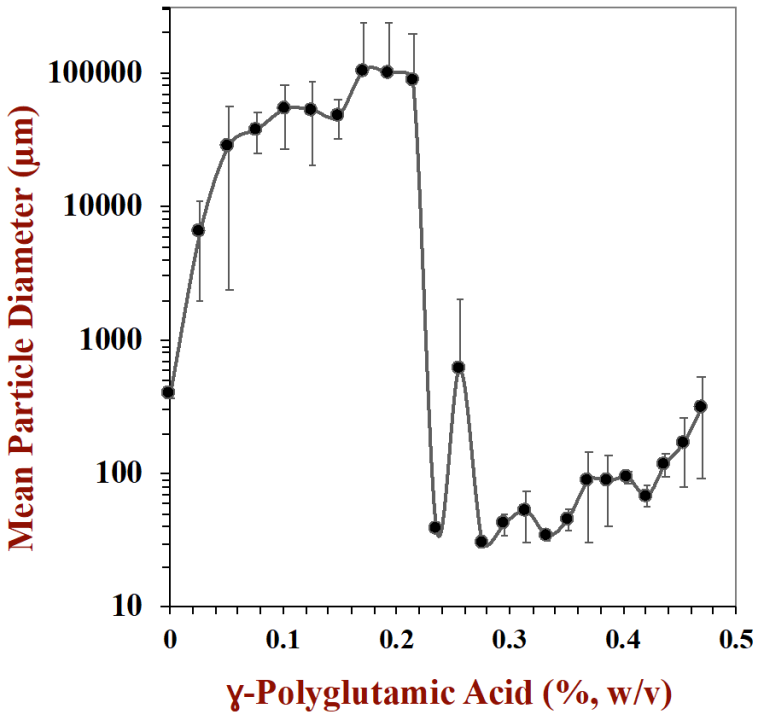
Figure 2.4. Dependence of the turbidity (550 nm) of 0.5% w/w poly-L-lysine solutions when increasing concentrations of poly-glutamic acid were added (pH 7.4). The inset shows photographs of the different samples.

This result suggests that insoluble electrostatic complexes were formed at intermediate ɣ-PGA levels. Interestingly, the maximum turbidity was observed at the same ɣ-PGA level as the point of zero charge **(Figure 2.4)**, which suggests that the complexes may have aggregated due to a reduction in the electrostatic repulsion between them. At low ɣ-PGA concentrations, the complexes had a positive charge that prevented them from aggregating due to a strong electrostatic repulsion, whereas at high ɣ-PGA concentrations they had a negative charge that also prevented them from aggregating.

Particle size analysis

Particle size measurements were carried out using dynamic light scattering to provide some additional insights into the nature of the insoluble complexes formed at intermediate ɣ-PGA levels. In agreement with the turbidity measurements, the mean particle diameter first increased with increasing ɣ-PGA concentration, but then decreased at higher levels (**Figure 2.5a**), which suggested that large aggregates were formed at intermediate polyglutamate levels. The particle size distribution measurements indicated that the insoluble aggregates formed at intermediate ɣ-PGA levels (0.21%) had a monomodal distribution with a peak around 250 nm **(Figure 2.5b)**.

(A)



(B)

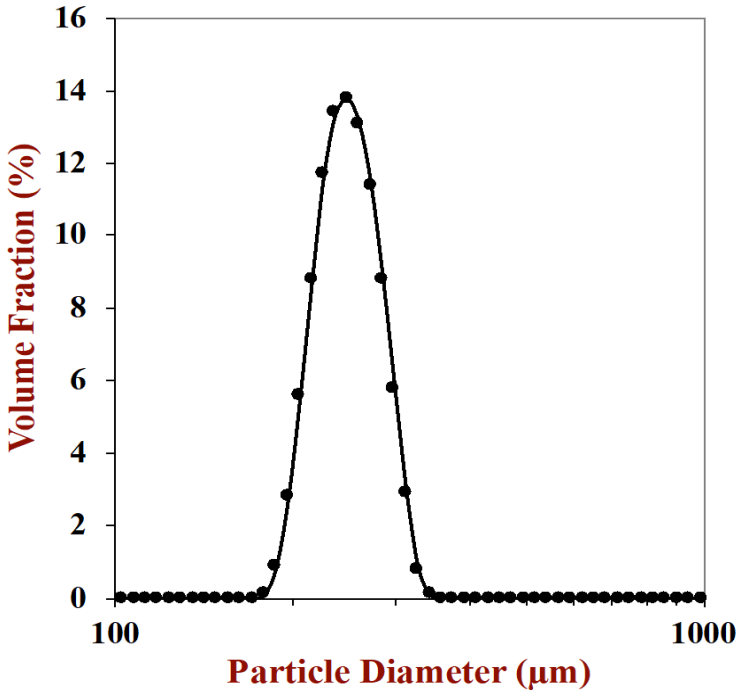


Figure 2.5: (A) Dependence of the mean particle diameter (ζ-average) of 0.5% w/w poly-L-lysine solutions when increasing concentrations of poly-glutamic acid were added (pH 7.4). (B) Particle size distribution of sample containing 0.5% w/w poly-L-lysine and 0.21% poly-glutamic acid (pH 7.4). Both were measured with dynamic light scattering.

Interaction enthalpy analysis

The enthalpy changes associated with the interactions between ɣ-PGA and ε-PLL were quantified using ITC (pH 7.4, 25°C). Two experiments were performed: (i) ɣ-PGA was injected into ε-PLL solution; (ii) ɣ-PGA was injected into water (**Figure 2.6**).

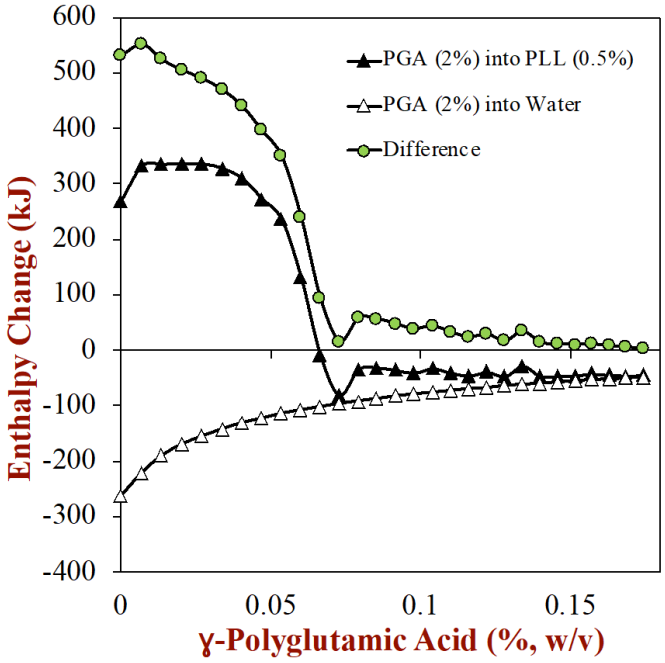


Figure 2.6. Enthalpy changes resulting from injecting increasing amounts of a 2% polyglutamic acid solution into either pH-adjusted water or a 0.5% w/w poly-L-Lysine solution (pH 7.4). The enthalpy changes were measured using isothermal titration calorimetry.

Injection of ɣ-PGA into water led to a strong exothermic enthalpy change, whose magnitude decreased as increasing levels of ɣ-PGA were added. This effect can be attributed to the heat of dilution of the ɣ-PGA. Initially, the ɣ-PGA molecules in the injector are quite close together, which leads to strong intermolecular interactions between them, presumably due to electrostatic repulsion. When they are injected into the reaction chamber, they are diluted and move further apart from each other, which changes the strength of the molecular interactions and leads to an exothermic reaction. Conversely,

injection of ɣ-PGA into the ε-PLL solution led to a strong endothermic enthalpy change initially, followed by a slight exothermic enthalpy change later, which is indicative of a strong interaction between the two polyelectrolytes.

The interaction between the ɣ-PGA and ε-PLL was assessed by calculating the difference between the two ITC profiles (**Figure 2.6**). There was a relatively large endothermic enthalpy change from around 0 to 0.05% ɣ-PGA, which decreased steeply when the polyglutamate level was further increased. There appeared to be a relatively small endothermic enthalpy change from around 0.08 to 0.15% ɣ-PGA. At sufficiently high ɣ-PGA levels, the enthalpy change for the sample (injecting ɣ-PGA into ε-PLL solution) was similar to that of the control (injecting ɣ-PGA into water), so the overall enthalpy change was close to zero. These results suggest that the two oppositely charged polyelectrolytes interacted strongly with each other at low ɣ-PGA levels, but then all the positively charged groups on the polylysine became saturated, which meant that there was little further interaction. Interestingly, the concentration range where the strong interactions were observed by ITC (0 to 0.07 %) was considerably narrower than the range where there was a large increase in turbidity and particle size (0 to 0.2%)[133-134]. This suggests that electrostatic complexes may have formed first (strong endothermic reaction), which then aggregated with each other to produce large insoluble complexes (weak endothermic reaction). In future studies, it would be interesting to identify the precise molecular origin of the observed enthalpy changes, *e.g.,* electrostatic attraction, counter-ion release, conformational changes of polypeptides (such as secondary structure formation), and aggregation. This was, however, beyond the scope of the current work.

2.2.2.2: Complex stability

In this series of experiments, we examined the impact of solution conditions, such as pH, salt, and temperature, on the stability of the electrostatic complexes formed by the polylysine and polyglutamic acid.

Influence of pH

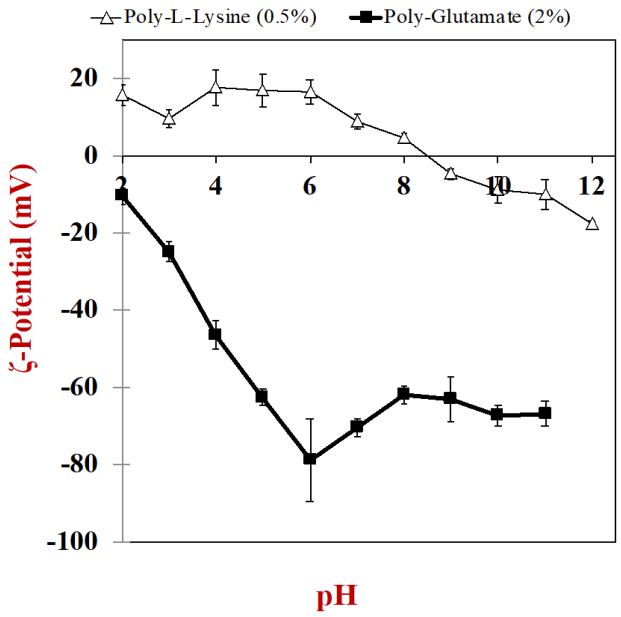


Figure 2.7. Dependence of the ζ-potential of aqueous solutions of poly-L-lysine (0.5%) and poly-glutamic acid (2%) on pH measured using particle electrophoresis.

The pH of the aqueous phase alters the electrical characteristics of the polyelectrolytes, which should impact their ability to form electrostatic complexes. For this reason, the impact of solution pH on the properties of the electrostatic complexes formed from ɣ-PGA and ε-PLL was examined.

The surface potential (ζ-potential) of the polyelectrolytes and their complexes was measured at different pH levels using microelectrophoresis **(Figure 2.7)**. The surface potential (ζ-potential) of the ε-PLL went from moderately positive at low pH to moderately negative at high pH, with a point of zero charge around pH 8.5 (**fig. 2.7**). This effect can be attributed to the different charged groups on the polylysine. Additionally, Polylysine has numerous amino groups in the side chains, as well as terminal carboxyl and amino groups on either end of the molecule [130, 135]. The positive charge at low pH values can therefore be attributed to protonation of the amino groups ($–NH3^+$) and carboxyl groups (–COOH). Conversely, the negative charge at high pH values can be attributed to the de-protonation of the amino groups (–NH2) and carboxyl groups ($–COO^-$). The polyglutamate was negatively charged across the whole pH range studied, but the magnitude of the negative charge decreased appreciably when the pH was reduced from around pH 6 to 2. This effect can be attributed to protonation of the carboxyl groups under acidic conditions (–COOH), since the pKa value of these groups has been reported to be around 4.9 [114, 136]. In this case, the impact of the terminal amino group on the charge characteristics is much less important because the polyglutamate has a much higher molecular weight than the polylysine [130]. Based on the ζ-potential versus pH profiles, one would expect the two polyelectrolytes to form complexes with each other over a wide range of pH values since they have opposite charges from pH 2 to 8.3.

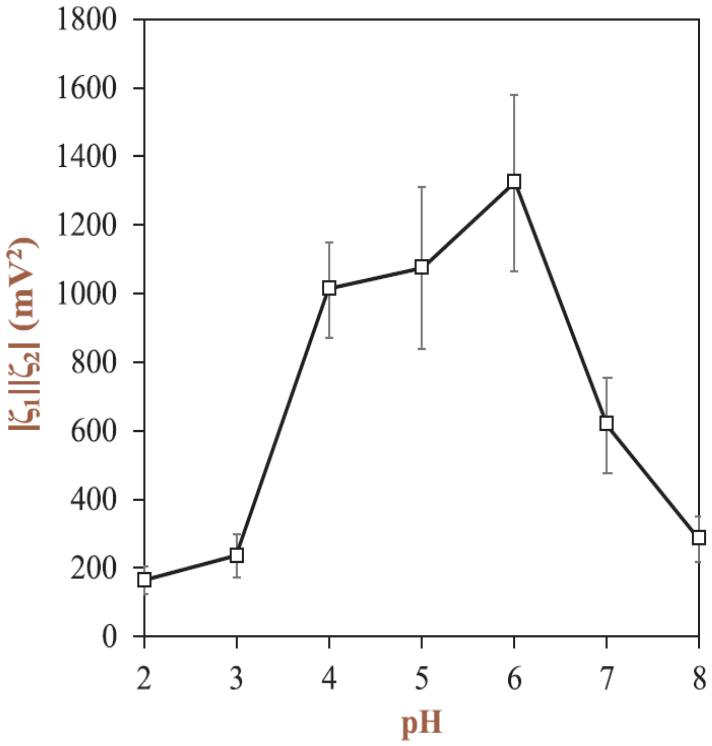


Fig 2.8. Dependence of the product of the absolute values of the ζ-potentials ($|\zeta_1|\times|\zeta_2|$) of the poly-L-lysine (0.5%) and poly-glutamic acid (2%) solutions on pH. This curve suggests that the strongest electrostatic attraction occurs around pH 6.

An estimate of the magnitude of the electrostatic attraction between the two polyelectrolytes was obtained by plotting the absolute values of the ζ-potentials ($|\zeta_1|\times|\zeta_2|$) of the poly-L-lysine and poly-glutamic acid solutions as a function of pH (**Figure 2.8**). This analysis suggested that the strongest electrostatic attraction between the two polyelectrolytes should occur around pH 6.

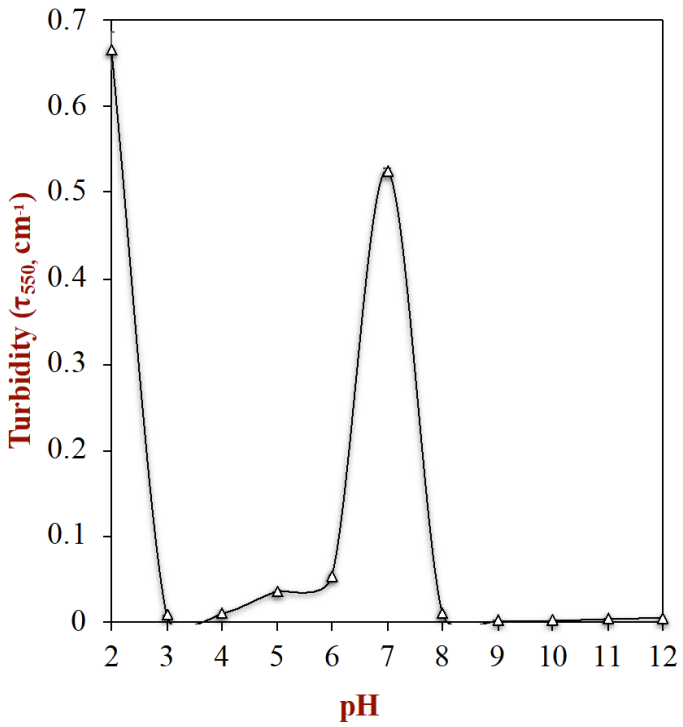


Figure 2.9. Dependence of the turbidity of aqueous solutions of mixed poly-L-lysine (0.5%) and poly-glutamic acid (0.235%) on pH.

The impact of pH on the turbidity of mixtures consisting of 0.5% polylysine and 0.235% polyglutamic acid was then measured (**Figure 2.9)**. The mixtures were optically transparent from pH 12 to 8, which can be attributed to the fact that both polyelectrolytes had a net negative charge (**Figure 2.7**). Hence, there was an electrostatic repulsion between them that inhibited their tendency to form complexes. There was a relatively high turbidity from pH 8 to 5, which can be attributed to the formation of electrostatic complexes that were large enough to strongly scatter light. These complexes formed because of the relatively strong electrostatic attraction between the oppositely charged polyelectrolytes in this pH range (**Figures 2.7 and 2.8**). From pH 4 to 3, the turbidity of the mixed

polyelectrolyte solutions was relatively low suggesting that large electrostatic complexes were not formed. This was probably because of the relatively weak electrostatic attraction between the polylysine and polyglutamic acid in this pH range (**Figures 2.7 and 2.8**). Interestingly, there was a large increase in turbidity at pH 2 (**Figure 2.10)**, which was probably due to self-association of the polyglutamic acid when the carboxylic acid groups became protonated, thereby reducing the electrostatic repulsion between the molecules.

Influence of Salt

These presence of mineral ions in aqueous solutions can alter the formation and stability of electrostatic complexes due to their ability to bind to polyelectrolytes and to screen electrostatic interactions [105, 108, 127]. For this reason, we examined the impact of salt (NaCl) on the properties of the electrostatic complexes formed by ɣ-PGA and ε-PLL.

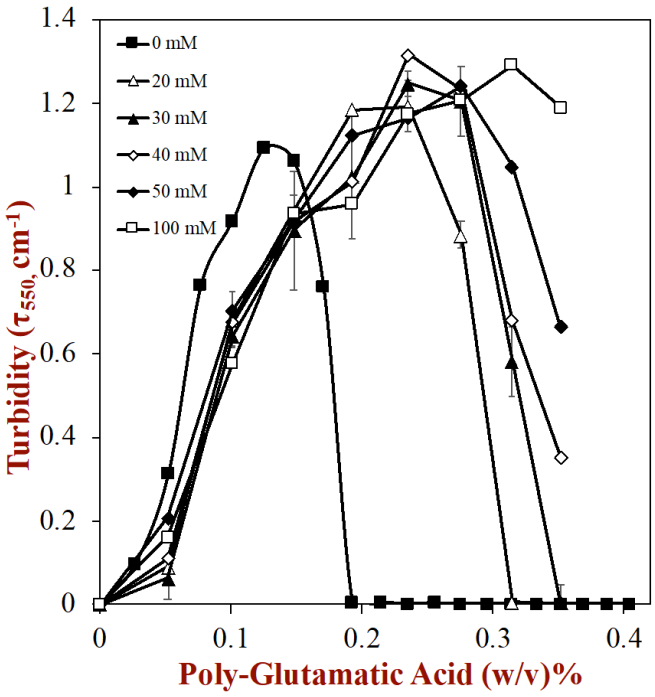


Figure 2.10: Impact of salt (NaCl) concentration on the dependence of the turbidity (550 nm) of 0.5% w/w poly-L-lysine solutions when increasing concentrations of poly-glutamic acid are added (pH 7.4).

There was a broadening of the range of ɣ-PGA concentrations where large electrostatic complexes that scattered light strongly was formed as the salt concentration was increased **(Figure 2.10)**. This suggests that the aggregates were more susceptible to aggregation in the presence of salt, which can be attributed to screening of the electrostatic repulsion between them at higher ionic strengths. This result suggests that the complexes formed between the two polyelectrolytes were highly susceptible to changes in the ionic composition of the surrounding aqueous phase, which may have important implications for their practical application in food products.

Influence of temperature

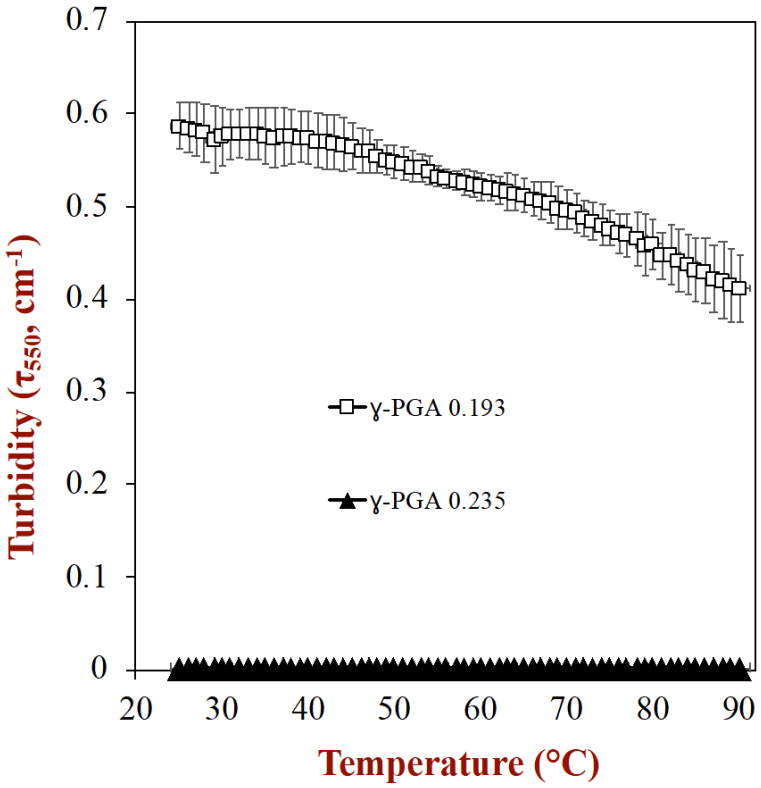


Figure 2.11. Impact of temperature on the turbidity (550 nm) of 0.5% w/w poly-L-lysine solutions containing either 0.193% (insoluble complexes) or 0.235% (soluble complexes) poly-glutamic acid (pH 7.4).

Finally, the impact of temperature on the thermal stability of electrostatic complexes was measured using turbidity measurements **(Figure 2.11)**. Two mixed samples were selected for analysis based on the turbidity measurements made at ambient temperature **(Figure 2.4 or 2.11)** (*see below*): (i) insoluble complexes that were highly turbid (γ-PGA = 0.193%; ε-PLL = 2.0 w/v%); (ii) soluble complexes that were transparent (γ-PGA = 0.235%; ε-PLL = 2.0 w/v%). The turbidity of the sample containing insoluble

complexes decreased slightly as the temperature was raised from 25 to 90 °C, which suggests that there was limited dissociation of the polyelectrolyte complexes at higher temperatures. This phenomenon may have occurred due to alterations in the strength of the hydrogen bonding or electrostatic interactions with increasing temperature, as well as alterations in the configurational and conformational entropy. Even so, the complexes did remain intact during heating, which suggests that the electrostatic attraction was strong enough to hold the two polyelectrolytes together. The turbidity of the sample containing soluble complexes remained low at all temperatures indicating that heating did not lead to the formation of insoluble complexes. This might be important for the development of products that remain clear during thermal processing.

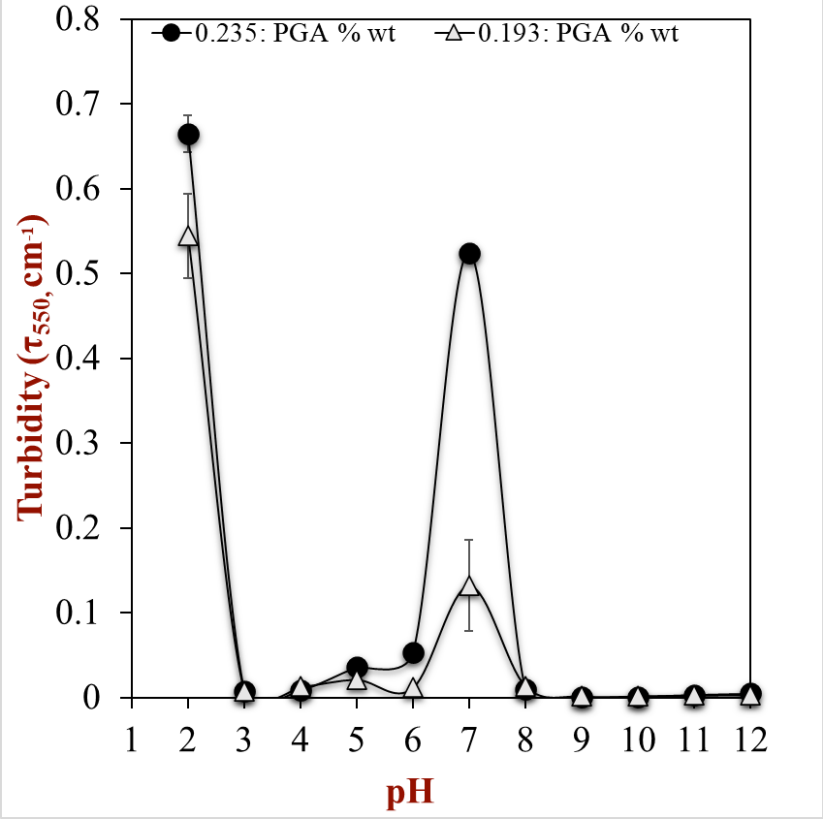


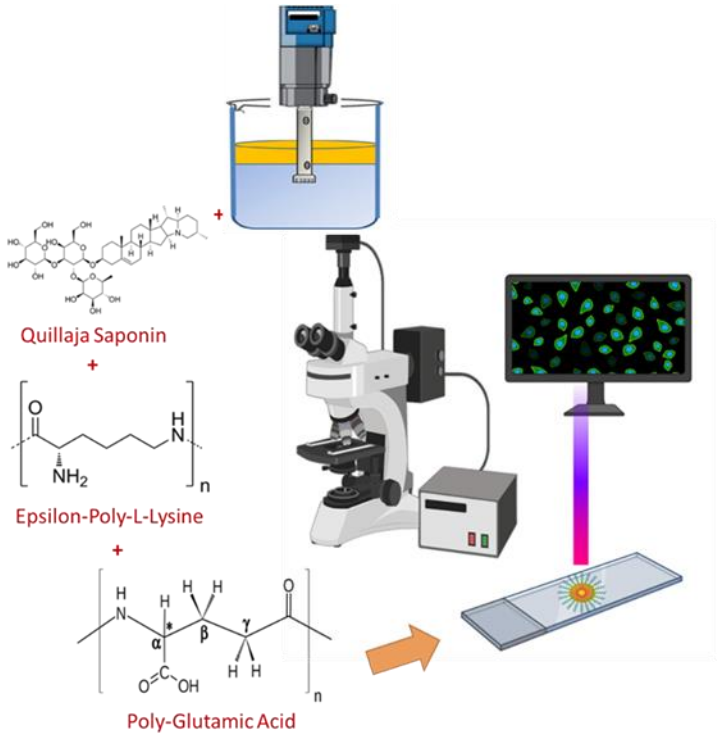
Figure 2.12. Samples selected from turbidity (550 nm) experiments of 0.5% w/w poly-L-lysine solutions containing either 0.193% (insoluble complexes) or 0.235% (soluble complexes) poly-glutamic acid (pH 7.4).

2.3 Conclusions

Complex coacervation is a useful approach for creating biopolymer-based colloidal particles for the oral delivery of bioactives, such as nutraceuticals, vitamins, and pharmaceuticals. In this study, we examined the possibility of using anionic ɣ-poly-glutamic acid (PGA) and cationic ε-poly-L-lysine (PLL) to form polyelectrolyte complexes. Initially, the formation and properties of the complexes were characterized using visual observations, UV-visible spectrophotometry, microelectrophoresis (ζ-potential), and isothermal titration calorimetry (ITC) measurements. The impact of pH, ionic strength, temperature and polymer ratio on complex formation was examined. Our results showed that the complexes formed had a 1:4 mass ratio of polyanion-to-polycation at saturation. These results may be useful for the design of effective oral delivery systems for bioactive agents in foods and other products. This study has also shown that cationic polylysine and anionic polyglutamic acid can be used to assemble electrostatic complexes in aqueous solutions. These two polyelectrolytes can be produced by microbial fermentation and may therefore be used as natural biopolymers for creating novel structures and functionalities in foods. For instance, they may be used to encapsulate, protect, and release bioactive agents. We have shown that the formation and properties of the complexes is dependent on pH, ionic strength, and temperature, which can be attributed to the importance of electrostatic interactions and hydrogen bonding in their formation. In future studies, we intend to utilize these complexes to form colloidal delivery systems for bioactive agents.

CHAPTER 3. STABILIZATION OF SOYBEAN OIL-IN-WATER EMULSIONS USING POLYPEPTIDE MULTILAYERS: CATIONIC POLYLYSINE AND ANIONIC POLYGLUTAMIC ACID.

Table of Content:



3.1 Introduction

Many lipid-rich foods are in the form of oil-in-water emulsions such as milk, soft drinks, nutritional beverages, dressings, soups, and sauces [87]. Typically, the oil phase is dispersed as small spherical lipid droplets that are coated by a single layer of emulsifier molecules. This kind of emulsion, however, is often highly susceptible to aggregation, especially when exposed to stresses such as heating, refrigeration, dehydration, pH extremes, and high salt levels [23, 137-139]. In addition, there is only limited scope to tailor the functional attributes of lipid droplets that are stabilized by a single-layer coating. The functional performance and stability of emulsified systems can be controlled by engineering

the composition and structural organization of the coatings around the lipid droplets [25]. Indeed, previous studies have shown that emulsions containing lipid droplets coated by multiple layers can have much better stability than those coated by single layers [3, 13, 21, 82]. This kind of interfacial engineering has been used to create emulsion-based delivery system with enhanced physical and chemical stability [23, 28].

Multilayer coatings can be assembled from food-grade emulsifiers and biopolymers that are charged, like ionic surfactants, phospholipids, proteins, or polysaccharides [3, 8, 21, 23, 77, 82]. Droplets coated by multilayers have been employed to encapsulate hydrophobic flavors [88, 140], ω-3 fatty acids [141], vitamin A [2], and carotenoids [25, 142]. The electrostatic deposition method is therefore a versatile tool for modulating the functional performance of emulsion-based delivery systems by intelligently altering their interfacial properties. The assembly conditions and biopolymers utilized to create multilayer coatings around lipid droplets impact the stability and functionality of emulsions [3, 54]. Typically, a polypeptide and an oppositely charged polysaccharide are used to fabricate multilayer coatings from food-grade biopolymers [77]. In the current study, we examined the possibility of forming these coatings using two oppositely-charged polypeptides. These polypeptides were selected because of their well-defined primary sequence. Epsilon-poly-L-lysine (PLL) is a cationic polypeptide that is positively charged across a wide pH range because of the relatively highly dissociation constant of the primary amino groups, *i.e.,* pK_a =10 [125, 143]. Conversely, poly-glutamic-acid (PGA) is an anionic polypeptide that is negatively charged across a broad pH range because of the relatively low dissociation constant of the primary carboxyl groups, *i.e.,* pK_a = 4.9 [114, 130]. Recently, we showed that PLL and PGA form either insoluble or soluble electrostatic complexes in aqueous solutions under appropriate pH and salt conditions [18].

PLL-PGA complexes were insoluble under pH conditions where there was a strong electrostatic attraction between them, whereas they were soluble when there was only a weak attraction. In the current study, we hypothesized that that the ability of the two polypeptides to form electrostatic complexes under appropriate solution conditions could be utilized to form polypeptide-based multilayer coatings around lipid droplets, which may improve the physicochemical stability and functional performance of emulsions.

Initially, primary emulsions were formed by homogenizing soybean oil, water, and a natural surfactant (quillaja saponin) together. These emulsions contained anionic saponin-coated lipid droplets, which were then used as a template to form the multilayer-coated droplets. Secondary emulsions were formed by depositing a layer of cationic PLL onto the surfaces of the saponin-coated droplets. Finally, tertiary emulsions were formed by depositing a layer of anionic PGA onto the PLL-saponin-coated droplets. The impact of changes in environmental conditions on the stability of the primary, secondary, and tertiary emulsions was then measured. Our main objective was to determine whether there were any significant advantages to coating the lipid droplets with multilayers of polypeptides.

3.2 Materials and Methods

Materials

Epsilon-poly-L-lysine (2.5 to 3.8 kDa) (CAS No. 73548-20-60) and ɣ-poly-glutamic acid (≥ 700 kDa) (CAS No. 25513-46-6) were purchased from Wilshire Technologies Inc. (Princeton, NJ, USA). Prior to utilization the powdered ingredients were stored at ambient temperature under dry conditions. Quillaja saponin (Q-Naturale 200) was provided by National Starch LLC (Bridgewater, N.J.). Soybean oil was acquired from a local supermarket.

All other chemicals were of analytical grade. Double distilled water from a water purification system (NANOpure Infinity, Barnstead International, Dubuque, IA) was used to prepare all solutions.

Delivery system	Size Range	Appearance	Main composition	Advantage	Disadvantage	References
Micelle	5-100 nm	Optically transparent	Amphiphilic compound (emulsifier)	• Thermodynamic stable colloidal stable • High loading capacity of encapsulated compound • Easy to prepare	• Low long-term holding capacity of encapsulated compound • High amount of amphiphilic compounds, required for the preparation • Undesirable final taste	144
Liposome	25 nm – 2.5 µm	Optical transparent – opaque	Phospholipids	• Able to encapsulate both hydrophilic and lipophilic nutraceutical simultaneously. • Nanosize liposomes can be thermodynamic stable, but kinetically unstable. • Natural ingredients	• Low stability at low acidic pH condition • High material costs • Low solubility	145

Microemulsion	5-50 nm	Optical transparent	Amphiphilic compounds and liquid oil	• Thermodynamic stable • Fast release • Easy to prepare	• High amount of amphiphilic compound	146
Nanoemulsion	50-100 nm	Transparent or translucent	Amphiphilic compounds and liquid oil	• medium surfactant required. • fast release • Can be prepared by using natural ingredients. • Relatively low cost.	• Thermodynamic unstable • Tend to break over time, under gravitational separation, flocculation, coalescence, Ostwald ripening and phase inversion.	16, 146
Emulsion	>100 nm	Opaque	Amphiphilic compounds and Liquid oil	• Lowest surfactant required. • Fast release • Can be prepared by using natural ingredients. • Relatively low cost.	• Thermodynamic unstable • Tend to break over time, under gravitational separation, flocculation, coalescence, Ostwald ripening and phase inversion.	16

Solid Lipid particles	1-1000 nm	Depends on particle size. (from transparent to opaque)	Amphiphilic compounds and solid lipid phase	• Low lipid oxidation and encapsulated chemical degradation rate than lipid oil. • Able to encapsulate both lipophilic and hydrophilic. • Good holding drug inside of particles.	• Temperature sensitive • Large particle Thermodynamic unstable • Low drug loading capacity • Dispersion in high water content	145, 147
Hydrogel particles	100 nm - micrometers	Originally Clear Gel-like Transparency depends on loaded compound	Gelling protein and/ carbohydrate	• No surfactant required • High loading capacity • Prepared using natural ingredients. • Target release	• Porous structure • High Density-Sedimentation • Poor internal material release rate • pH or/and temperature sensitive	148

Table #3.1. The size range, appearance, main compositions, advantages, and disadvantages of different colloidal delivery systems.

Emulsion Fabrication

Oil-in-water emulsions were produced by homogenizing 10 w/v% lipid phase (soybean oil) with 90 w/v% aqueous phase (0.5% quillaja saponin in distilled water adjusted to pH 4.0). Initially, a coarse emulsion was formed by applying a laboratory blender (Bamix, Biospec Products, Bartlesville, OK) for 2 minutes at ambient temperature. The coarse emulsion was then poured into the inlet hopper of a microfluidizer (M110-P, Microfluidics, Newton, MA, USA). The sample was passed through this device three-times at a pressure of 15,000 psi. An ice bath was placed around the collection vessel to cool the emulsion and prevent the microfluidizer from over-heating during the homogenization process.

Formation of Multilayer Coatings

Secondary emulsions: After homogenization, the soybean oil-in-water emulsions ($d \approx 0.50$ μm) were adjusted back to pH 4.0. A series of secondary emulsions was then formed by mixing the primary emulsion with PLL solutions of varying concentration. Practically, this was achieved by injecting 2 mL of primary emulsion (as five aliquots of 0.4 mL) into a fixed volume (8 mL) of PLL solution (0-0.02 w/v%) while vortexing so as to reach a final sample volume of 10 mL. The final lipid droplet concentration in the secondary emulsions was therefore 2.0 w/v% while the PLL concentration increased from 0 to 0.016 w/v%. The PLL-to-water (pH 4.0) ratio in the PLL solutions was varied to generate a range of poly-L-lysine concentrations in the final emulsions. The secondary emulsions were then stored for another 24 h to allow the PLL to full adsorb to the lipid droplet surfaces.

Tertiary emulsions: A series of tertiary emulsions was formed by mixing the secondary emulsions with PGA solutions of varying concentrations. This was achieved by adding 5 mL of secondary emulsion (2 w/v% oil) into 0 to 2 mL of PGA solution (0.08 w/v%) while vortexing to give a range of PGA concentrations from (0 to 0.023 w/v%). The tertiary emulsions were then incubated at ambient temperature for 24 h to allow the system to come to steady state.

Influence of Environmental Conditions

The influence of pH, salt, and heating on the stability of the emulsions was examined. For these measurements, the primary, secondary, and tertiary emulsions were all diluted with pH-adjusted distilled water or salt solution so that they had the same final oil droplet concentration (1.0 w/v%).

- *pH:* The impact of pH was examined by preparing emulsions using double distilled water adjusted to different pH values (pH 2.0-9.0) and then vortexing to ensure they were homogenous.

- *Ionic Strength:* Emulsions (pH 4.00) containing a range of salt concentrations were prepared by mixing stock primary, secondary, or tertiary emulsions with different ratios of 500 mM NaCl solution (pH 4.00) and water (pH 4.0) and then vortexing.

- *Heating:* Primary, secondary, or tertiary emulsions (pH 4.00) were placed on a temperature-controlled water bath (Fisherbrand™ Isotemp™ Advanced Stirring Hotplate, Fisher Scientific) and held at temperatures from 30 to 90°C for 30 min. After heating, they were cooled to ambient temperature.

After each of these treatments, the emulsions were stored for 24 h at ambient temperature before being analyzed.

Determination of surface potential and size of coated lipid droplets

Microelectrophoresis and light scattering were used to determine the surface potential and dimensions of the coated lipid droplets in the different emulsions. The ζ-potential was measured by electrophoresis (Zetasizer Nano-ZS, Malvern Instruments, Malvern, Worcestershire, UK) while the mean particle diameter (d_{32}) was measured by laser diffraction (MasterSizer 3000, Malvern Instruments). Each sample was diluted with double distilled water or salt solution (adjusted to the appropriate pH) prior to analysis so as to reach the optimum light scattering conditions for reliable measurements. In the salt-addition experiments, the emulsions were diluted with salt solutions (pH 4.0) that had the same ionic strength as them for the ζ-potential measurements but with distilled water (pH 4.0) for the particle size measurements (because of the large diluent volume required). All measurements were conducted on at least two freshly prepared samples and repeated three times per sample.

Microstructural analysis

Optical and confocal scanning fluorescence laser microscopy were used to monitor the microstructures of the emulsions using a 40×objective lens (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, US). For the fluorescence measurements, 100 μL of sample was mixed with 10 μL of Nile Red solution (1 mg/mL ethanol) to dye the lipid phase. The emission and excitation wavelengths used for the Nile Red dye were 543 nm and 605 nm, respectively. Images were collected and stored using the image analysis software on the microscope (NIS-Elements, Nikon, Melville, NY).

Statistical analysis

All data were collected from at least two separate experiments with two measurements made per sample. The results were then combined, and the mean and standard deviation were calculated using Excel (Microsoft, Redmond, VA, USA).

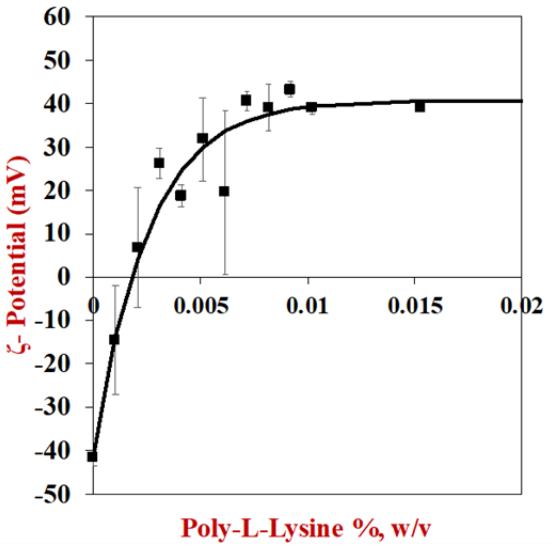
3.3: Optimization of Primary Emulsion Formation

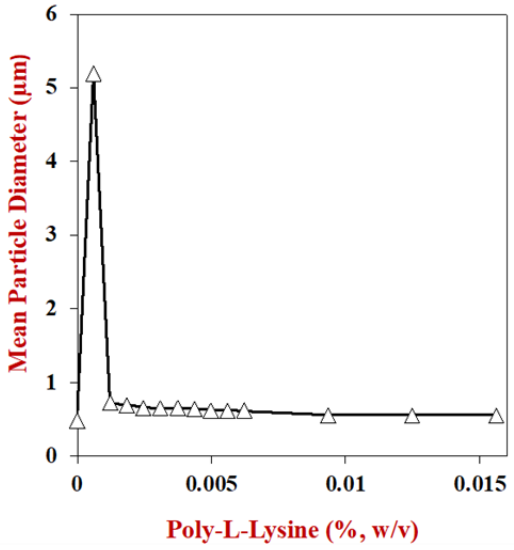
Initially, a preliminary experiment was carried out to establish the optimum emulsifier concentration required to create the primary emulsions. The emulsifier concentration should be sufficiently high to generate small lipid droplets during homogenization but not so high that there is a high level of non-adsorbed emulsifier present in the aqueous phase. This excess emulsifier would have interacted with the cationic polypeptide (PLL) used to form the secondary layer, thereby inhibiting multilayer formation. For this reason, an initial experiment was conducted that involved measuring the impact of emulsifier concentration on the mean particle diameter and ζ-potential of 10 w/v% soybean oil-in-water emulsions produced under standardized homogenization conditions **(data not shown)**. These experiments established that a mean droplet diameter of 0.50 μm and a ζ-potential of -41.9 mV could be obtained using 0.5 w/v% of quillaja saponin. For this reason, these conditions were used in the remainder of the experiments.

3.4: Optimization of Secondary Emulsion Formation

The purpose of these experiments was to identify the optimum (Epsilon-Poly-L-Lysine) PLL concentration required to completely cover the surfaces of the saponin-coated lipid droplets. The adsorption of the cationic PLL molecules onto the anionic saponin-coated lipid droplets is mainly driven by electrostatic attraction between the polypeptide chains and the surfaces of the oppositely charged lipid droplets. Typically, solution conditions are selected so that both the polypeptides and lipid droplets have strong surface potentials but opposite charges [3, 149]. It is important, however, that the final surface potential of the polypeptide-coated lipid droplets is large enough to generate a strong electrostatic repulsion, otherwise the droplets will aggregate. In this study, we found that pH 4.0 was a suitable value for achieving these goals.

(A)





(B)

(C)

Figure 3.1. Impact of PLL concentration on the (A) ζ-potential, (B) mean particle diameter (d_{32}), and (C) appearance of oil-in-water emulsions containing lipid droplets coated by quillaja saponin (pH 4.0). In the photographs, the PLL concentration increases from 0 to 0.016% (left to right), corresponding to the values in Figs. 2.1A and 2.1B.

The ζ-potential changed from strongly negative (-42 mV) in the absence of PLL, to strongly positive (+44 mV) in the presence of high levels of PLL **(Fig. 3.1a)**. The net charge on the coated droplets was close to zero at a PLL concentration of around 0.0013 w/v%, suggesting that the cationic groups on the PLL molecules had neutralized all of the exposed anionic groups on the adsorbed saponin molecules at this point. It should be noted that there were relatively large error bars for some of the ζ-potential measurements, which can mainly be attributed to the formation of large aggregates at PLL concentrations where charge neutralization or bridging flocculation occurred. The mean particle diameter was relatively small ($d \approx 0.50$ μm) in the absence of PLL and the droplets were stable to creaming, suggesting quillaja saponin was an effective natural emulsifier **(Figs. 3.1b and 3.1c)**. At an intermediate PLL concentration, which was quite close to the point of zero charge, there was a large increase in mean particle diameter ($d > 5$ μm), which is indicative of aggregation of the droplets. Presumably, this was because the net charge on the lipid droplets was relatively low, so only a weak electrostatic repulsion operated between them. Moreover, the surfaces of these droplets would have had both positive patches (PLL) and negative patches (saponin) exposed to the surrounding aqueous phase, thereby leading to an electrostatic attraction between them that could promote heteroaggregation. At higher PLL concentrations, however, the mean particle diameter was relatively small, decreasing from around 0.73 to 0.56 μm as the PLL level was raised from 0.0012 to 0.0025 w/v%. A

possible explanation of this result is that the PLL layer formed around the lipid droplets became thinner as the PLL concentration was raised. An alternative explanation is that the presence of higher levels of PLL inhibited droplet flocculation. Assuming that no droplet flocculation occurred at the highest PLL concentration, the thickness of the PLL layer can be estimated to be around 30 nm, *i.e.,* half the difference between the diameters of the coated (560 nm) and uncoated (500 nm) droplets. Visual observations of the secondary emulsions showed that they were all stable to creaming, except for the ones where extensive droplet flocculation was observed at intermediate PLL concentrations **(Fig. 3.1b)**.

More information about the characteristics of the interfacial coatings formed by the poly-L-lysine molecules was obtained by analyzing the ζ-potential *versus* PLL curve **(Fig 3.1a).** The following semi-empirical equation was fitted to the experimentally measured ζ-PLL profile [8]:

$$\frac{\Delta\zeta(c)}{\Delta\zeta_{Sat}} = \frac{\zeta(c) - \zeta_{Sat}}{\zeta_0 - \zeta_{Sat}} \approx \exp\left(-\frac{c}{c^*}\right) \approx \exp\left(-\frac{c}{3c_{Sat}}\right) \quad (1)$$

Here ζ_0, $\zeta(c)$, and ζ_{Sat} are the measured ζ-potential values of the lipid droplets in the absence of PLL, at a PLL concentration of c, and when they are saturated with PLL. The variable c^* is the PLL concentration where the overall change in ζ-potential is $1/e^{th}$ of the total change in the ζ-potential at saturation: $\Delta\zeta = (\Delta\zeta_{Sat})/e$. The variable c_{Sat} is the minimum PLL concentration needed to completely cover the droplet surfaces, which is calculated by assuming that the lipid droplet surfaces are saturated when the overall change in ζ-potential is 95% of Δζ: $c_{Sat} = -c^*\ln(0.05)$ or $c_{Sat} \approx 3c^*$ [8]. The value of c_{Sat} was estimated by

finding the best-fit between the predictions of the above equation and the measured ζ-potential *versus* PLL profile (**Fig. 3.1a)**. These calculations indicated that the c_{Sat} value was around 0.0075 w/v%. If it is assumed that all of the PLL binds strongly to the lipid droplet surfaces (below saturation) because of the strong electrostatic attraction between the cationic polypeptides and anionic droplet surfaces, then the surface load was determined to be 0.31 mg m^{-2}, which is relatively low compared to most proteins, *i.e.,* 1 to 10 mg m^{-2} [16]. This calculation is based on knowledge of the lipid droplet concentration (2% oil) and the mean droplet diameter (d_{32} = 0.5 μm). Knowledge of the amount of a polyelectrolyte required to saturate the surfaces of all the droplets in an emulsion is important because it means that multilayer emulsions can be formed using the *saturation method*, *i.e.,* just enough polyelectrolyte can be added to completely cover the droplet surfaces, without promoting bridging or depletion flocculation [3].

3.5: Optimization of Tertiary Emulsion Formation

Tertiary emulsions were formed by depositing anionic ɣ-PGA onto the cationic PLL-saponin-coated lipid droplets. These experiments were carried out at pH 4.0 since the carboxylic acid groups on PGA have a negative charge at this pH, while the amino groups on PLL have a positive charge [18]. A PLL concentration of 0.0096 w/v% was used to formulate the secondary emulsions because this level was expected to lead to a system where all the PLL was adsorbed to the lipid droplet surfaces, *i.e.,* slightly above the saturation concentration. As a result, there would be little free PLL present in the aqueous phase to interact with the PGA.

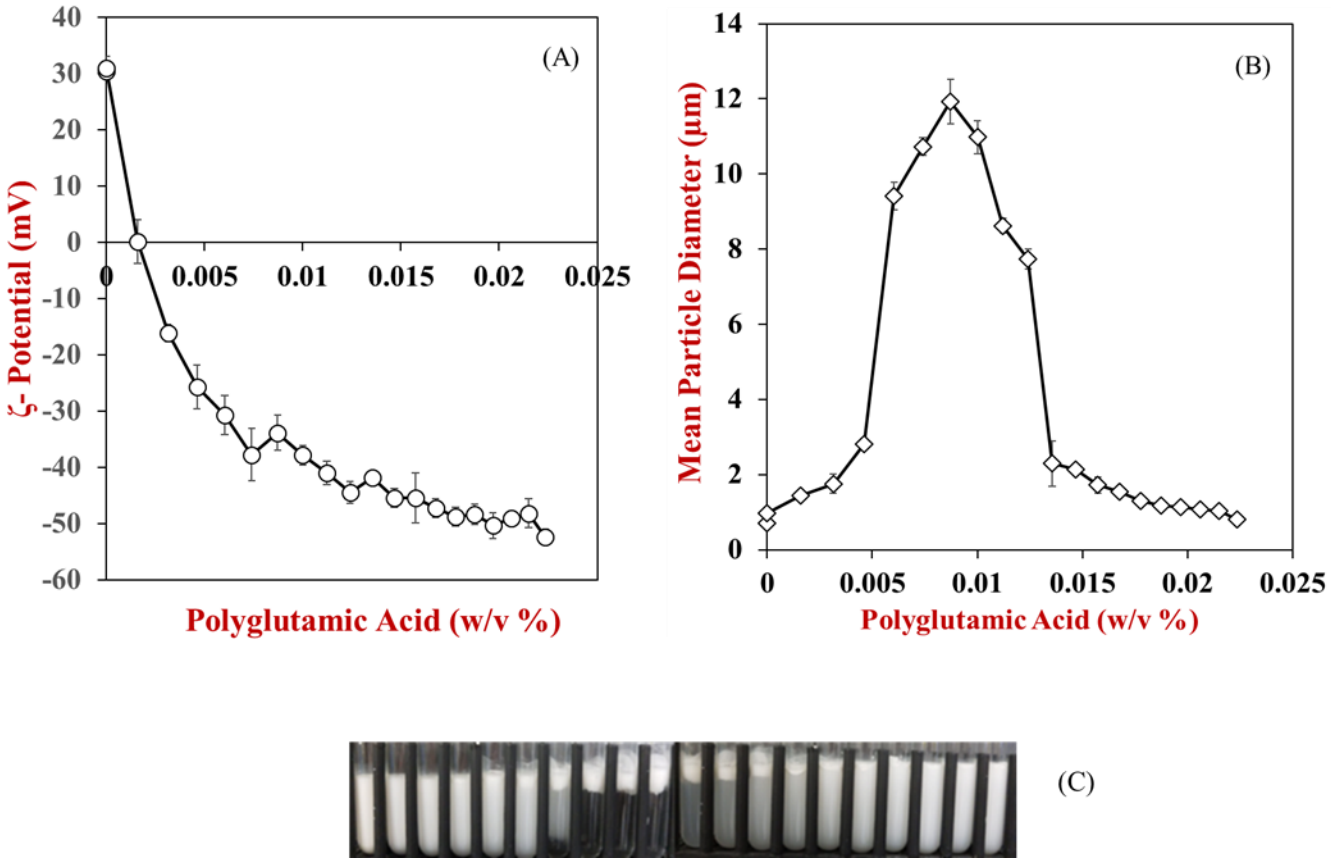


Figure 3.2. Impact of polyglutamic acid concentration on the (A) ζ-potential, (B) mean particle diameter (d_{32}) and (C) appearance of tertiary emulsions containing PLL-saponin-coated lipid droplets (pH 4.0). In the photographs, the PGA concentration increases from 0 to 0.022% (left to right), corresponding to the values in Figs. 2.2A and 2.2B.

The ζ-potential of the droplets in the tertiary emulsions was measured as the PGA concentration was raised **(Fig 3.2a)**. The ζ-potential went from strongly positive (+30 mV) in the absence of PGA to strongly negative (-50 mV) in the presence of high levels of PGA. This change in charge from cationic to anionic is indicative of the PGA molecules adsorbing onto the surfaces of the PLL-saponin-coated lipid droplets. The lipid droplets had a net zero

charge around 0.0031 w/v% PGA, which suggested that the negative groups on the PGA molecules had neutralized the positive groups on the PLL molecules around this value. The emulsion stability was also strongly dependent on PGA addition (**Figs. 3.2b** and **3.2c**). There was a steep increase in mean particle diameter as the PGA concentration increased from 0 to 0.01 w/v%, followed by a steep decrease when the PGA concentration was further increased to 0.031 w/v% (**Fig. 3.2c**). Indeed, relatively small droplets (d_{32} = 0.8 μm) could be formed at the highest PGA concentration used (0.023 w/v%). The particle size results were supported by visual observations of the tertiary emulsions, which showed that appreciable creaming occurred at intermediate PGA concentrations, but that the systems were relatively stable to gravitational separation at low and high PGA levels (**Fig. 3.2b**). The increase in particle size and creaming instability are indicative of droplet aggregation. The most likely mechanism for this phenomenon is bridging flocculation caused by linking of a single anionic PGA molecule to cationic patches on two or more cationic PLL-saponin-coated lipid droplets. Interestingly, the largest particle size and creaming instability did not occur at the point where the oil droplets had no net charge.

* In the subsequent experiments, a PGA concentration of (0.021 w/v%) (that was slightly above the saturation level) was used to formulate the tertiary emulsions.

3.6: Factors effecting properties of layers

There are many endogenous factors on a food matrix, but at the same time, exogenous from a multilayer system perspective, that usually effect on the external in a particular multilayer system. Here we are only going to focus on a few of the most

predominant factors that can be found in food processing facilities, such as *pH, Ionic Strength* and *Temperature*. The understanding of each effect caused by all the factors mention below, are imperative for the selection

pH

The properties of the multilayered interfaces surrounding colloidal particles can be adjusted by small variations in pH after their formation[86]. Alterations in solution pH can change the electrostatic interactions between the particle surface and the adsorbed polyelectrolyte, between two or more adsorbed polyelectrolytes in the layer, or between adsorbed and non-adsorbed polyelectrolytes. These changes can alter the thickness, packing and integrity of the multilayered interface[3]. For example, changing external pH conditions can be used to cause one or more of the polyelectrolytes in the interface to fall off, thereby providing a means of selective triggering of release of charged molecules[150]. Finally, pH induced alterations in the thickness of the interfacial layer may provide better or worse stability to droplet aggregation by altering the magnitude and range of the steric repulsion and van der Waals attraction between droplets[3].

Ionic Strength

The ionic strength of the solution determines the strength and range of intra- and inter-molecular electrostatic interactions and hence multilayer film formation, structure and thickness[4, 151]. In the presence of salt, polyelectrolytes often form thicker layers because they have a more compact chain conformation in solution (due to weaker intra-molecular repulsion) and because the weaker electrostatic attraction between charged polyelectrolyte

and surface groups allows post-adsorption molecular rearrangements[6]. Interestingly, electrostatic screening becomes stronger as the concentration and valency of the counter-ions in the solution increases. The range of this effect is characterized by the *Debye screening length* (κ−1), which varies with the inverse square root of the ionic strength:

$$\kappa^{-1} = \sqrt{\frac{\varepsilon_0 \varepsilon_R \mathrm{kT}}{e^2 \sum n_{0i} z_i^2}}$$

(*Debye screening length* (κ−1) for aqueous solutions at room temperature) here where:

κ^{-1} = 0.304/√ I nm, where I is the ionic strength of 1:1 electrolytes (e.g., NaCl) in moles per liter[152]. Multivalent counter-ions (e.g., Ca^{2+}, Fe^{2+}, Fe^{3+}) are much more effective at screening electrostatic interactions than monovalent counter-ions (e.g., Na^{+}, Cl^{-}, K^{+}). Thus, smaller concentrations of multivalent counter-ions would screen more charges than monovalent counter-ions. Multivalent counter-ions can also bind to the surface of polyelectrolytes and change the surface charge density[3, 14]. Generally speaking, polyelectrolyte molecules within the inner layers tend to be more densely packed and have more restricted motion than those in the outermost layer[153]. Therefore, the amount of polyelectrolyte adsorbed to the surface and the thickness, structure and porosity of the interfaces formed are strongly determined by the polyelectrolyte's sensitivity to its environment, e.g., pH, ionic strength and temperature[3].

Temperature (Heating)

Many food emulsions undergo some form of thermal processing during their production, storage or utilization, e.g., pasteurization, sterilization or cooking[16]. It is indeed imperative that an emulsion can withstand these thermal treatments without breaking down due to droplet flocculation or coalescence[20]. However, many emulsifiers are unsuitable for creating droplets that are resistant to thermal processing because they undergo changes in their ability to prevent droplet aggregation with temperature[3].

3.7: Impact of Environmental Conditions on Emulsion Stability

In these experiments, the stability of primary, secondary, and tertiary emulsions to environmental stresses (*pH, Ionic Strength* and *Heating)* were examined at pH 4.0. All the emulsions were diluted to a final oil level of 1.0 w/v% so that they could be compared at a similar droplet concentration. The particle size, charge, creaming stability, and microstructure were measured.

pH

Initially, the impact of pH on the properties of the primary, secondary, and tertiary emulsions was investigated. All of the emulsions were initially prepared at pH 4.0, and then they were adjusted to a range of different pH values by adding acid or alkaline solution. The particle charge, size, and creaming stability were then measured after 24 h incubation at ambient temperature **(Fig. 3.3)**.

Interestingly, the sign of the ζ-potential of all the emulsions was independent of pH: the primary and tertiary emulsions were always negative, while the secondary emulsions were always positive **(Fig. 3.3a)**. The magnitude of the ζ-potential of the primary emulsions was also largely pH-independent. There was an appreciable decrease in the magnitude of the positive charge on the secondary emulsions under highly acidic conditions (pH 2 and 3). Moreover, there was a slight decrease in the negative charge on the tertiary emulsions under the most acidic conditions. As mentioned earlier, the dissociation constants of the amino groups on the PLL should be around $pK_a = 10$, whereas those of the carboxyl groups on the PGA should be around $pK_a = 4.9$. Consequently, one would expect the PLL to remain strongly positively charged across the whole pH range, but some loss of negative charge on the PGA molecules when the system was reduced below pH 5. The reduction in negative charge of the tertiary emulsions observed at low pH may therefore have been due to partial protonation of the carboxyl groups and/or desorption of some of the polypeptides in the outermost layer of the lipid droplets.

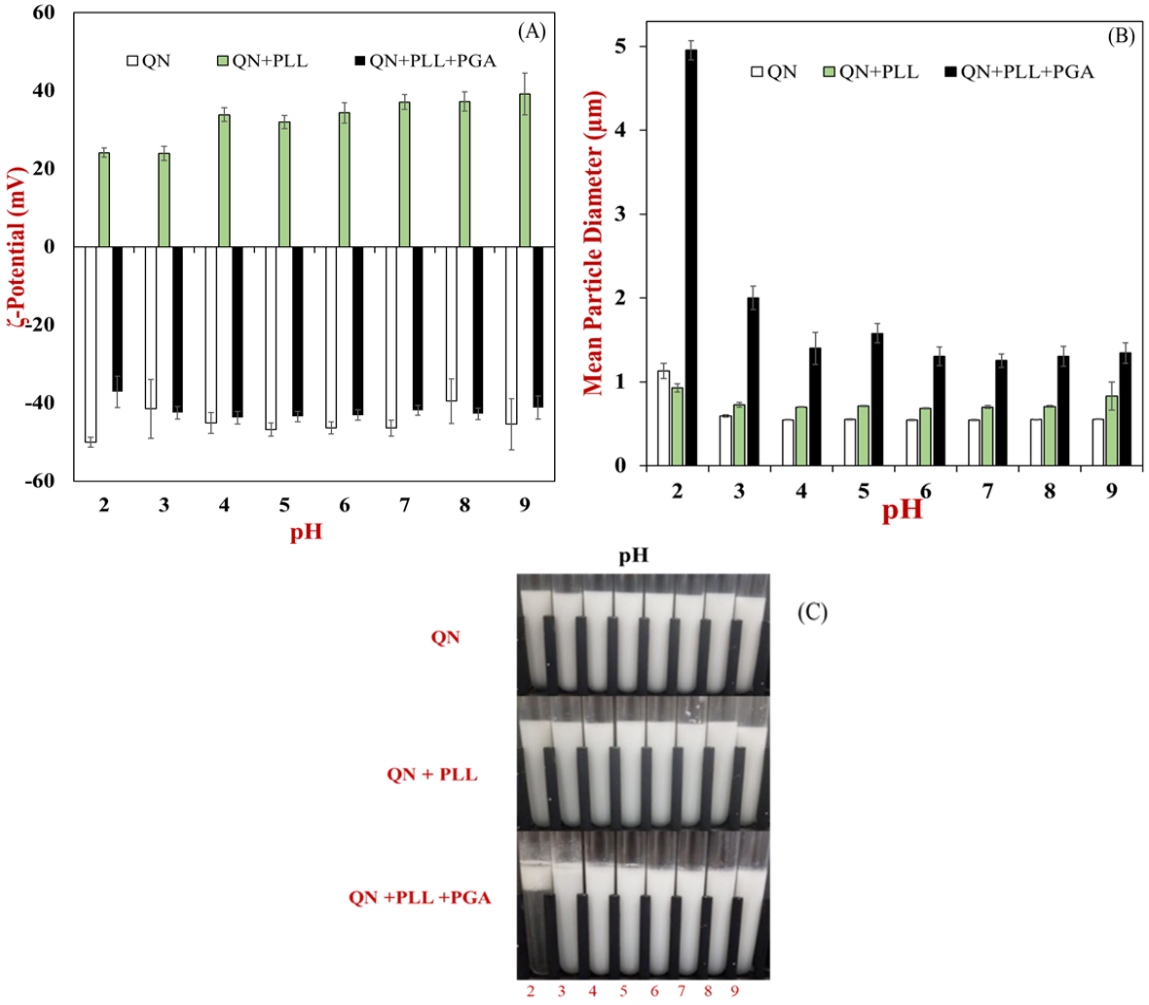


Figure 3.3. (A) ζ-Potential, (B) mean particle diameter (d_{32}) and (C) appearance of 1st, 2nd and 3rd layers systems pH influence from (2.00-9.00)

The size of the particles in the emulsions was also pH-dependent **(Fig. 3.3b)**. The mean particle diameter of the primary emulsions remained relatively low from pH 3 to 9, but increased appreciably at pH 2. These results suggest that droplet aggregation occurred

in the primary emulsions under highly acidic conditions. Previous researchers have also reported that lipid droplets coated by quillaja saponin flocculate at low pH values [154], which was attributed to a reduction in the electrostatic repulsion between the droplets under strongly acidic conditions.

The *secondary emulsions* contained slightly bigger particles that the primary emulsions at most pH values, which may have been due to the presence of the adsorbed PLL layer or because of some flocculation. The secondary emulsions did, however, appear to be more stable to aggregation under highly acidic conditions than the primary emulsions.

The *tertiary emulsions* clearly had the worst pH stability. They contained the largest particles at all pH values, and a large increase in mean particle diameter occurred when they were incubated under highly acidic conditions. This phenomenon may have occurred because the PGA molecules lost some of their charge and so did not adhere to the lipid droplet surfaces as strongly. As a result, some of them became partially detached from the surface of one lipid droplet and adsorbed on to the surface of another lipid droplet, leading to bridging flocculation.

The particle size measurements were supported by visual observations of the emulsions **(Fig. 3.3c)**. The primary and secondary emulsions, which contained relatively small particles, were relatively stable to creaming at all pH values. Conversely, the tertiary emulsions were stable to creaming at higher pH values (pH 4 to 9), but rapidly creamed at the lowest pH value (pH 2).

Ionic Strength

In this section, the impact of ionic strength on the characteristics of the droplets in the *primary, secondary*, and *tertiary* emulsions was measured **(Fig. 3.4)**. The ionic strength was varied by adding increasing amounts of sodium chloride to the aqueous phase of the emulsions. We hypothesized that the addition of salt would screen the electrostatic interactions between the charged polypeptides within the multilayers, as well as between the surfaces of the charged lipid droplets, thereby altering their surface characteristics and stability. The surface potential of the emulsions was highly dependent on salt concentration when they were diluted with a solution of the same ionic strength (**Fig. 3.4a**). For the primary and tertiary emulsions, the lipid droplets remained negatively charged at all salt concentrations, but the absolute value of the ζ-potential decreased with increasing ionic strength. This effect is the result of the accumulation of counter-ions (Na^+) around the negative groups on the droplet surfaces at high salt levels, which reduces the surface potential [16].

For the *secondary emulsions*: the sign of the surface potential changed from positive to negative when the NaCl concentration was increased from 0 to 50 mM. This suggests that the PLL layer detached from the droplet surfaces in the presence of even low levels of salt, which may have been due to weakening of the electrostatic attraction between the cationic PLL and anionic saponins. The critical condition for the adsorption of a polyelectrolyte chain onto an oppositely charged sphere in a salt solution is given by the following expression [155]:

$$\sigma_c \xi \sim \kappa^a.$$

Here, ξ is the linear charge density of the polyelectrolyte, σ is the surface charge density of the sphere, and κ is the reciprocal of the Debye length. The value of κ increases as the ionic strength increases. Consequently, an increase in ionic strength means that a polypeptide would need to have a higher charge density to stick to the surface of a charged sphere. In other words, the tendency for the cationic PLL to attach to the anionic saponin-coated lipid droplets decreases as the salt concentration increases.

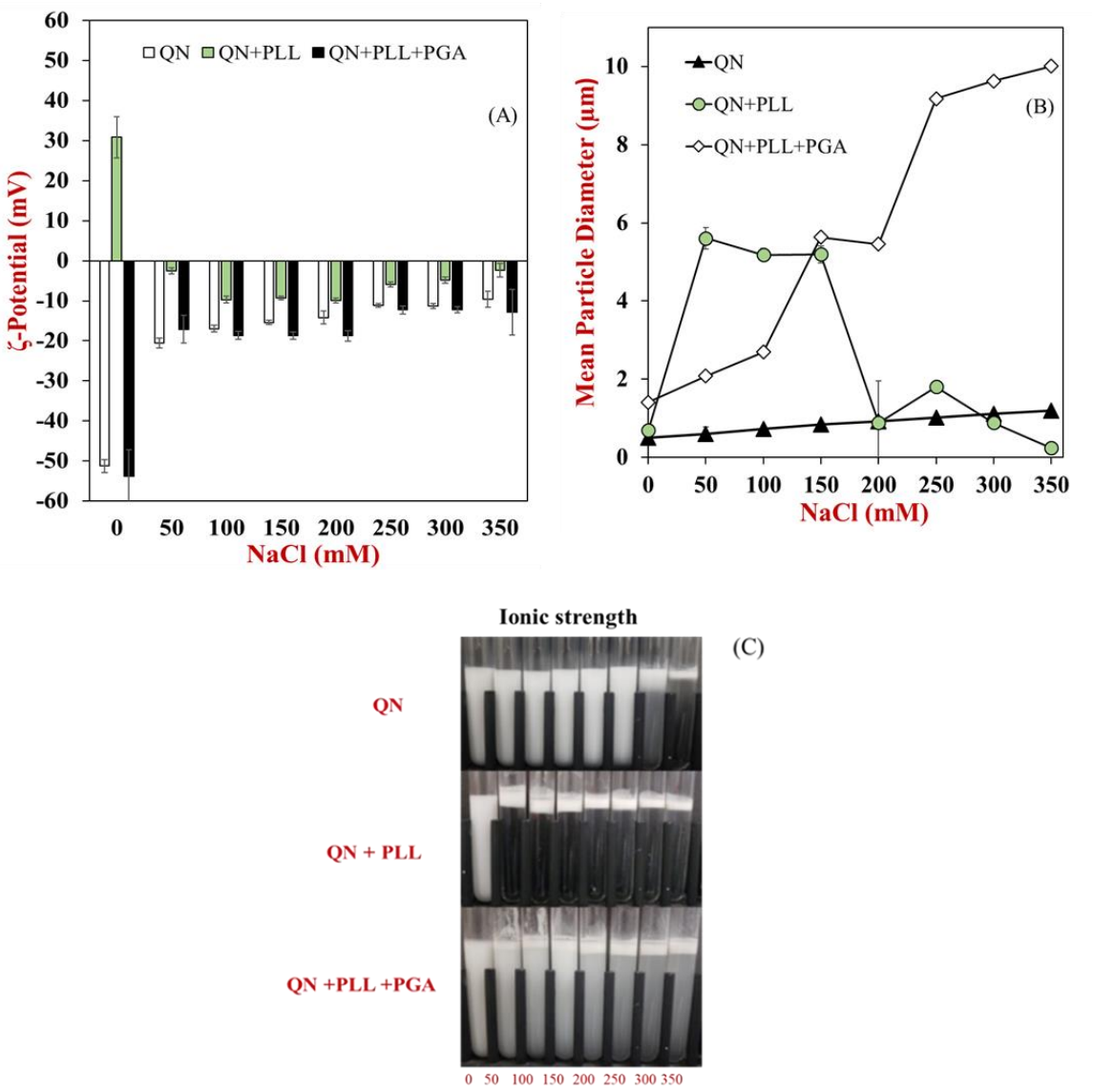


Figure 3.4. (A) ζ-Potential, (B) mean particle diameter (d_{32}) and (C) visual appearances of 1st, 2nd, and 3rd layers systems with respective Ionic Strength (0- 350mM) NaCl interactions.

The particle size measurements and visual observations of the emulsions indicated that they have very different salt-stabilities depending on the number of layers coating the

lipid droplets **(Figs. 3.4b and 3.4c).** For the *primary emulsions,* there was only a slight increase in mean particle diameter with increasing salt concentration, indicating that some aggregation of the lipid droplets had occurred. On the other hand, the visual observations of these emulsions indicated that extensive creaming occurred at the higher salt levels (300 and 350 mM), even though there was not a correspondingly large increase in particle size. The most likely reason for this result is that the lipid droplets were only held together by relatively weak attractive forces, and so the flocs were disrupted when they were diluted and stirred for the light scattering measurements. Previous studies with lipid droplets coated by quillaja saponins have also reported that they have relatively good salt-stability, which was attributed to a strong initial surface potential and some steric stabilization by the polar surfactant moieties [154].

For the *secondary emulsions,* the measured particle size was relatively large in the presence of low levels of salt (50-150 mM) but relatively small at higher levels. Conversely, visual observations of the secondary emulsions indicated that they were susceptible to creaming from 50 to 350 mM NaCl, suggesting they were flocculated at all salt levels. The most likely origin of this phenomenon is the change in electrostatic interactions with ionic strength. At low salt levels, the electrostatic repulsion between the droplets is weakened, which would allow them to approach more closely to each other. Moreover, the electrostatic attraction between the PLL and saponin molecules at the droplet surfaces would be weakened. As a result, a PLL molecule may partially detach from one droplet and partially attach to another droplet, thereby causing bridging flocculation to occur. At higher salt levels, the electrostatic attraction between the PLL and saponin molecules was very weak, so that the PLL molecules were largely detached from the droplet surfaces, which is

consistent with the charge reversal observed in the surface potential measurements. Even so, some of the PLL molecules may still have been weakly attached to the droplet surfaces due to weak electrostatic attraction between them and the saponin molecules. As a result, the droplets in the undiluted emulsions were weakly flocculated, leading to the observed creaming instability. However, these weak flocs were easily disrupted when the emulsions were diluted for the laser diffraction measurements, thereby leading to the decrease in particle size observed at higher salt levels.

In the *tertiary emulsions,* there was a steady increase in mean particle diameter with increasing salt concentration and evidence of extensive creaming at the higher salt levels. Thus, neither of the multilayer emulsions appeared to have good resistance to salt addition. As mentioned above, this may have been because the attractive forces between the outer polypeptide layer and the oppositely charged layer below may have been weakened by high salt levels. As a result, the polypeptide molecules could become attached to a number of different droplets, leading to bridging flocculation. Alternatively, the counter-ions from the salt may have weakened the electrostatic repulsion between the lipid droplets, leading to flocculation at higher salt levels.

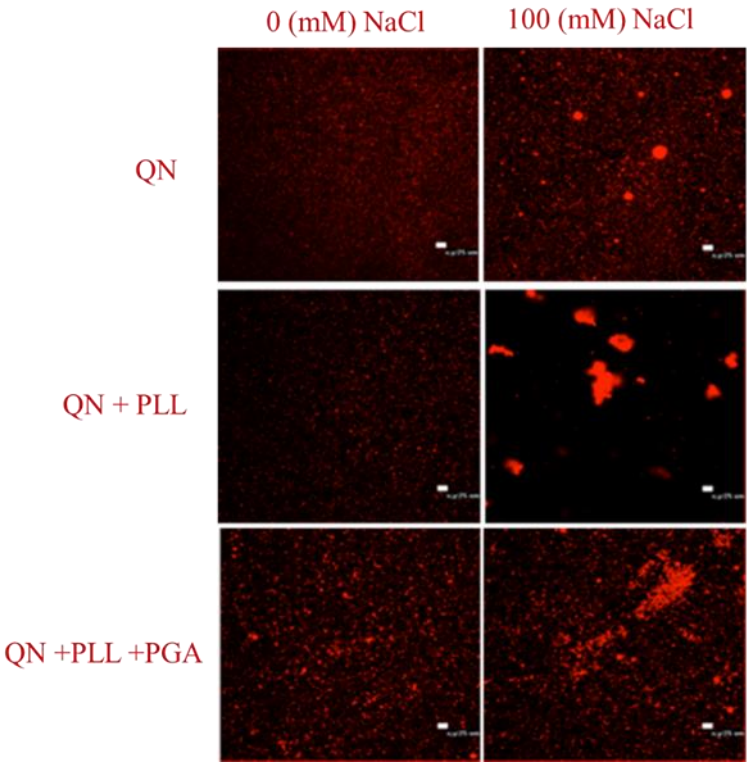


Figure 3.5. Confocal Microscopy of primary, secondary and tertiary layer with/without NaCl (100mM) interactions.

Analysis of the microstructure of selected emulsions (0 or 100 mM NaCl) supported the particle size and creaming stability measurements **(Fig. 3.5)**. For the primary emulsions, only a small fraction of the droplets had aggregated at the higher salt levels. Conversely, in the secondary emulsions, all of the droplets had aggregated and formed large dense particles. In the tertiary emulsions, a fraction of the lipid droplets appeared to be non-aggregated but another fraction appeared to be aggregated into large open particles.

Temperature

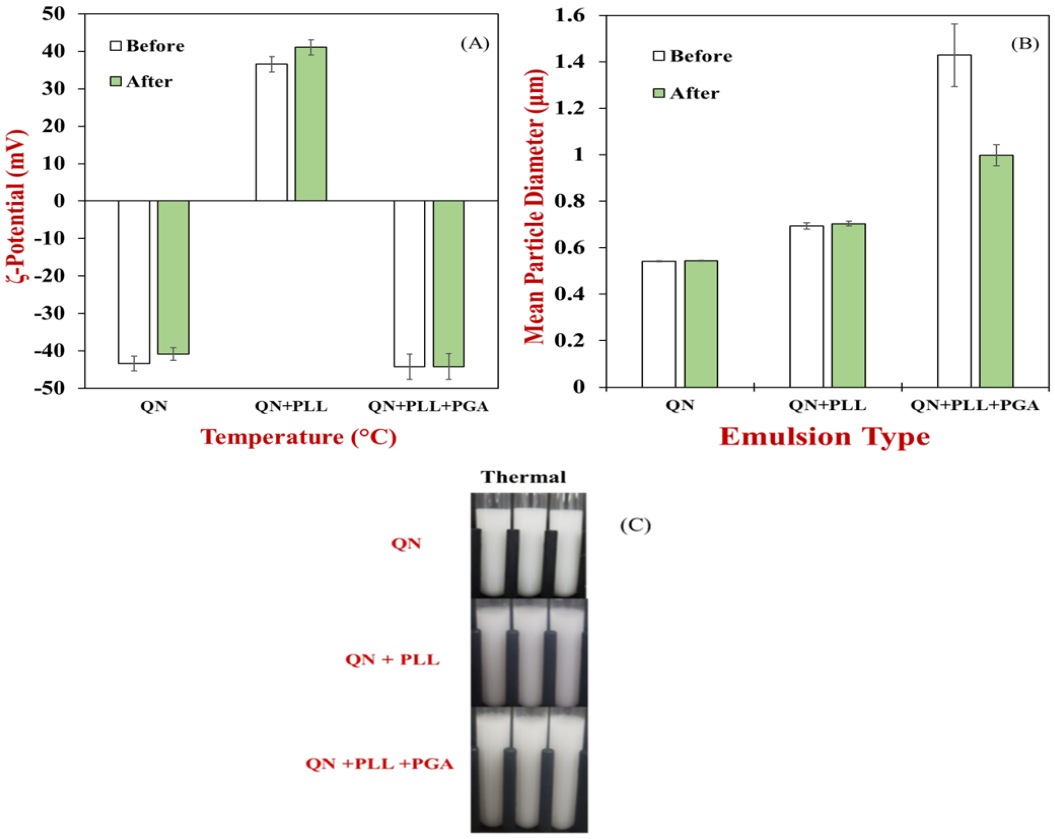


Figure 3.6. (A) ζ-Potential, (B) mean particle diameter (d_{32}) and (C) appearance of 1st, 2nd, and 3rd layers systems "Before vs After" Thermal-Heating exposure.

The thermal stability of the emulsions (pH 4.0) was also measured by heating them to different temperatures (30-90 °C) for 30 minutes, cooling them to ambient temperature, and then measuring their particle characteristics **(Fig. 3.6a-b)**. There was little change in the ζ-potential, size, or creaming stability of the particles in any of the emulsions, with the exception of the tertiary emulsion, in which there was an appreciable decrease in particle size after heating. This decrease suggests that there was some breakdown of the flocs at

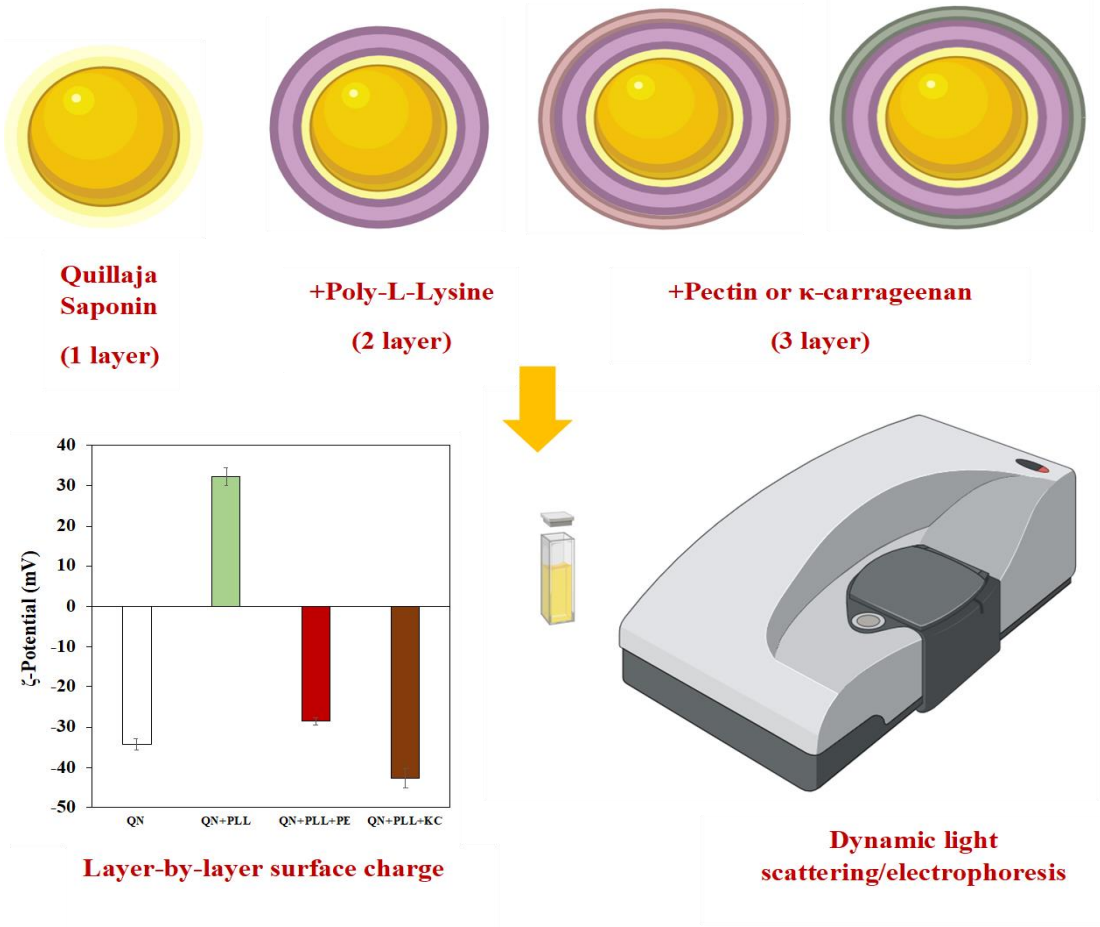
higher temperatures, possibly due to a weakening of the hydrogen bonding between the polypeptide chains. The particle size measurements were supported by visual observations of each emulsion **(Fig. 3.6c)**. Overall, these results indicate that the multilayer emulsions were relatively stable to heating, which may be important for some commercial applications.

3.8: Conclusions

Our original hypothesis was that the formation of polypeptide multilayers around lipid droplets would improve their stability to alterations in environmental conditions, such as pH, ionic strength, or temperature. Initially, we demonstrated that primary, secondary, and tertiary emulsions could be produced from anionic quillaja saponin, cationic poly-l-lysine (PLL), and anionic polyglutamic acid (PGA) using sequential electrostatic deposition. The lipid droplets coated by polypeptide multilayers, however, were less stable to pH, salt, and thermal processing than those simply coated by saponin monolayers. As the multilayer emulsions are more expensive to formulate and require more complicated processing operations, there would seem to be little advantage in using the layer-by-layer electrostatic deposition method with these polypeptides to improve the physical stability of the emulsions. Interestingly, however, the PLL layer on the secondary emulsions remained attached to the droplet surfaces at low salt levels but became detached at higher salt levels. This phenomenon may be utilized for certain specialized applications, *e.g.,* if it is an advantage to have cationic droplets within a food product but anionic ones inside the human body. In future studies, we intend to examine the impact of the polypeptide multilayers on the oxidative stability and digestibility of encapsulated lipids to determine whether there are some other specific applications where they may have advantages.

CHAPTER 4. ENHANCING EMULSION FUNCTIONALITY USING MULTILAYER TECHNOLOGY: COATING LIPID DROPLETS WITH SAPONIN-POLYPEPTIDE-POLYSACCHARIDE LAYERS BY ELECTROSTATIC DEPOSITION.

Table of Content:



4.1. Introduction

There has been great interest in improving or extending the functional properties of emulsions by coating the lipid droplets with coatings consisting of numerous layers of different biopolymers [13, 23, 48, 137]. The stability and functionality of these multilayer emulsions can be tuned by careful selection of the type, number, and sequence of

biopolymer molecules within the coatings. [13, 21]. Coating oil droplets using the layer-by-layer (LbL) deposition method has been shown to have many potential benefits for food applications, including the ability to control or trigger the release of encapsulated components, the ability to improve the resistance of emulsions to processing and storage conditions (such as pH changes, salt addition, heating, freezing, and dehydration), and the ability to modulate the digestibility of lipids within the gastrointestinal tract [3, 156-157]. Furthermore, multilayer coatings can be designed to retard lipid oxidation in emulsions due to their ability to alter both the charge and thickness of the interfacial layer around the oil droplets [141, 158]. The electrical properties of the first layer of a multilayer emulsion are determined by the emulsifier, and they can therefore be controlled by selecting different types of emulsifier [78-79]. These multilayer coatings can be assembled from food-grade charged emulsifiers and biopolymers, like ionic surfactants, phospholipids, proteins, or polysaccharides, using a sequential electrostatic deposition method [23, 80, 159-160]. However, the selection of the most appropriate combination of coating materials is critical to forming a stable system with the desired functional attributes [77].

In this study, we used an anionic plant-based food-grade surfactant, quillaja saponin (QS), to form the first layer around the lipid droplets. A cationic polypeptide, epsilon-poly-L-lysine (PLL), was then used to form the second layer because it has a strong positive charge across a wide pH range, since the amino groups have pK_a values around 10 [92, 118, 120, 125]. The ability of cationic PLL to coat anionic saponin-coated lipid droplets was recently demonstrated in our earlier study [161]. The resulting 2-layer emulsions contained cationic droplets, but they were highly unstable to aggregation when exposed to environmental stresses, such as pH changes and salt addition. This is because counterions, such as H^+, OH^-,

Na^+ or Cl^-, can bind to oppositely charged groups on the surfactants or polymers, thereby weakening the molecular interactions holding the multilayers together. For this reason, we examined the possibility of improving the functional performance of these 2-layer emulsions by further coating the droplets with an anionic polysaccharide using the saturation method [161]. In this method, just enough polysaccharide is included in the aqueous phase to completely cover all of the droplet surfaces, thereby leaving little free polysaccharide in the system. The presence of any free polysaccharide could interfere with the formation of any subsequent layers or promote depletion flocculation. We examined the potential of two different anionic polysaccharides, pectin (PE) and κ-carrageenan (KC), to form the outer layer around the lipid droplets.

Pectin is a branched polysaccharide with a linear backbone containing many ionizable carboxylic acid groups ($pK_a \approx 3.5$), as well as numerous "hairy regions" consisting neutral side chains extending from the backbone [45, 162]. κ-carrageenan is a linear polysaccharide with many sulfate groups ($pK_a \approx 2.0$) providing a strong negative charge across a broad range of pH values [47-48, 163]. We hypothesized that the ability of these two polysaccharides to form and stabilize multilayer emulsions would be different based on the distinct differences in their molecular characteristics. This knowledge could then be used to select the most appropriate polysaccharide to use in food applications where products are often exposed to alterations in pH, ionic strength, or temperature during their manufacture, storage or utilization.

Keywords: multilayers; poly-l-lysine; pectin; κ-carrageenan; polyelectrolytes; MCT O/W emulsions.

4.1 Materials and Methods

Materials

A medium chain triglyceride oil (Miglyol 812 N) was purchased from IOI Oleo GmbH (Witten, Germany). Quillaja saponin (Q-Naturale™ 200) was provided by National Starch LLC (Bridgewater, N.J.). The cationic polypeptide epsilon-poly-L-lysine (MW = 2.5 to 3.8 kDa) (CAS No. 73548-20-60) was purchased from Wilshire Technologies Inc. (Princeton, NJ, USA). The anionic polysaccharides (dietary fibers) were purchased from the Sigma Chemical Company (St. Louis, MO): pectin (from citrus peel) and κ-carrageenan (sulfated plant polysaccharide). All other chemicals were of analytical grade. Double distilled water obtained from a commercial water purification system (NANOpure Infinity, Barnstead International, Dubuque, IA) was used for the preparation of all solutions and emulsions.

Solution Preparation

All solutions were prepared at ambient temperature, unless stated otherwise. The aqueous emulsifier solutions were prepared by dispersing quillaja saponin (0.5-3.0% w/w) in distilled water adjusted to pH 4.0 and stirring for at least 1 hour. An aqueous PLL solution was prepared by dispersing 0.1% w/v of powdered epsilon-poly-L-lysine into double distilled water and then stirring continuously for at least 2 h. An aqueous pectin solution was prepared by dispersing 0.4% w/v of powdered pectin into distilled water and stirring for at least 2 h. Aqueous κ-carrageenan solutions were prepared by dispersing 0.3%, w/v of powdered carrageenan into distilled water and then boiling at 70°C for 20 min, cooling to ambient temperature, and then stirring for at least another 1 h. Finally, all of the biopolymer solutions were adjusted to pH 4.0 using NaOH and/or HCl solutions (0.1 M).

Emulsion droplet type	Equipment used	Parameters
Large	Hand-held blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland)	Low speed setting processed for 2 min
Medium	Ultrasonicator (model 500, Sonic Disembrator, Fisher Scientific, Pittsburgh, PA)	Frequency = 20 kHz amplitude = 20 %, duty cycle = 1 s processed for 2 min
Small	Air-driven microfluidizer (M110L, Microfluidics, Newton, MA)	Air pressure= 4 kpsi 1 pass
Very small	Air-driven microfluidizer (M110L, Microfluidics, Newton, MA)	Air pressure= 12 kpsi 3 passes

Table #4.1. Different emulsion sizes, and their respective preparation [16, 24, 164].

Emulsion Preparation

Emulsions were prepared using the same method described in our previous article [161], and so only a brief description is given here with any modifications described.

Primary Emulsions: Oil-in-water emulsions were prepared by homogenizing 10 wt% oil phase with 90 wt% aqueous emulsifier solution (pH 4.0). The oil and water phases were blended together using a high-shear mixer and then passed through a high-pressure microfluidizer (12,000 psi, 3 passes) to form small anionic saponin-coated lipid droplets. At the end of this process, the primary emulsions were adjusted back to pH 4.0, if required. Initially, a range of saponin concentrations (0.5-3.0% w/v) was used to establish the optimum emulsifier concentration to formulate the primary emulsions. The optimum saponin concentration (0.1% w/v) for further studies was then selected based on the ζ-potential and particle size data: this concentration gave small highly charged droplets at a relatively low emulsifier concentration.

Secondary Emulsions: Secondary emulsions were prepared by adding a fixed volume (2 mL) of primary emulsion (pH 4.0) into a fixed volume of (8 mL) of PLL solution (pH 4.0) with constant mixing. A series of PLL solutions with different polypeptide concentrations (0-0.025 %, w/v) were used in this experiment to establish the surface saturation concentration of the PLL. The final solutions therefore contained 2 w/v% oil droplets and 0 to 0.02 w/v% PLL. The secondary emulsions were then stored for 24 h to allow PLL to bind onto the QS-coated oil droplet surfaces.

Tertiary emulsions: Tertiary emulsions were formed by adding 5 mL of secondary emulsion (pH 4.0) to 5 mL of aqueous polysaccharide solution (pH 4.0) and then mixing. The polysaccharide solutions contained a range of pectin (0 to 0.2%) or κ-carrageenan concentrations (0 to 0.15%) so that the surface saturation concentrations of the polysaccharides could be established. The final systems contained 1 w/v% oil droplets and 0 to 0.075 w/v% (KC) or 0 to 0.080 w/v% (PE) polysaccharide. The tertiary emulsions were then incubated at ambient temperature for 24 h to allow them to come to steady state.

Environmental stress tests

Initially, the primary, secondary, and tertiary emulsions were diluted with pH-adjusted distilled water or salt solution (pH 4.0), so that they had the same final oil droplet concentration (1.0 w/v%). The influence of pH, salt addition, and heating on the stability of the emulsions was then examined using the same conditions described previously [161]. In summary, the emulsions were exposed to different pH values (2-9), salt concentrations (0-350 mM NaCl), and heating conditions (90°C, 30 min). After each treatment, the emulsions were stored for 24 h at ambient temperature before being analyzed.

Surface Potential and Particle Size Measurements

Microelectrophoresis and light scattering were used to determine the surface potential and particle size of the lipid droplets in the emulsions as described previously [161]. The ζ-potential was measured by electrophoresis (Zetasizer Nano-ZS, Malvern Instruments, Malvern, Worcestershire, UK) while the mean particle diameter was measured by laser diffraction (MasterSizer 3000, Malvern Instruments). All samples were diluted with

distilled water or salt solution (adjusted to the required pH) prior to analysis to ensure optimized light scattering conditions for reliable measurements. In the salt-addition experiments, the emulsions were diluted with salt solutions (pH 4.0) with the same ionic strength as for the ζ-potential measurements, but with distilled water (pH 4.0) for the particle size measurements (because of the large volume of water required). All measurements were conducted on at least two freshly prepared samples and repeated three times per sample.

Microstructural analysis

Confocal scanning fluorescence laser microscopy was used to characterize the structural organization of the lipid droplets within the emulsions (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, US). Prior to analysis, a fixed volume (200 μL) of emulsion was mixed with a fixed volume (10 μL) of Nile Red solution (1 mg/mL ethanol) to dye the oil droplets. An appropriate emission wavelength (543 nm) and excitation wavelength (605 nm) was used to detect the Nile Red.

Statistical analysis

All data were collected from at least three separate experiments with three measurements per sample. The results were then combined, and the mean and standard deviation calculated using Excel (Microsoft, Redmond, VA, USA).

4.3 Pectin

LM Pectin is a branched polysaccharide with a linear backbone containing many ionizable carboxylic acid groups ($pK_a \approx 3.5$), as well as numerous "hairy regions" consisting neutral side chains extending from the backbone. Pectin is not digested by gastric or small intestinal enzymes but is easily degraded by pectinases produced by the colonic microflora. LM pectin is more tolerant of pH variations and calcium levels, which could make it more suitable for colonic delivery systems formation. The charge characteristic of pectin can be selected to control lipid digestion and bioactive release, *e.g.*, decreasing DE reduced lipid digestion and bioactive bioavailability.

4.3.1 Pectin molecular and physicochemical characteristics

Chemical structure

Pectin linear **(fig 4.1a.)** backbone is mainly comprised of α(1-4)-d-galacturonic acid residues that may be partly esterified with methyl groups.

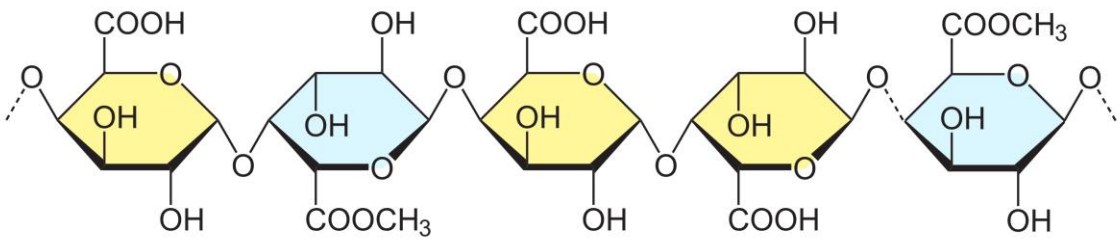


Figure 4.1a. Chemical Structure of Pectin

Polymer (Pectin) properties

Pectin is a well-known food additive which is mainly used for its gelling and stabilizing abilities. It is extracted from the plant cell wall, especially citrus peels, apple pomace and sugar beet pulps[45]. Pectin is a branched polysaccharide with a linear backbone containing many ionizable carboxylic acid groups ($pK_a \approx 3.5$) (isoelectric point around 3.5), as well as numerous "hairy regions" consisting neutral side chains extending from the backbone[45]. Isolated pectin can have a molecular weight typically of 60,000–130,000 g/mol, varying with origin and extraction conditions.

4.4 κ-carrageenan

In general Carrageenan's are natural anionic compounds that are normally extracted from red seaweeds [46]. However, κ-carrageenan is one of the most common forms of carrageenan's used in foods, and it is characterized by having D-galactose-4-sulphate, 3,6-anhydro-D-galactose-2-sulphate as a building block, and has a double-helix conformation[46, 48]. The popularity of this ingredient in the food industry is due to the ability of its linear helical portions to associate to form a three-dimensional gel in the presence of appropriate cations.

4.4.1 κ-carrageenan molecular and physicochemical characteristics

Chemical structure

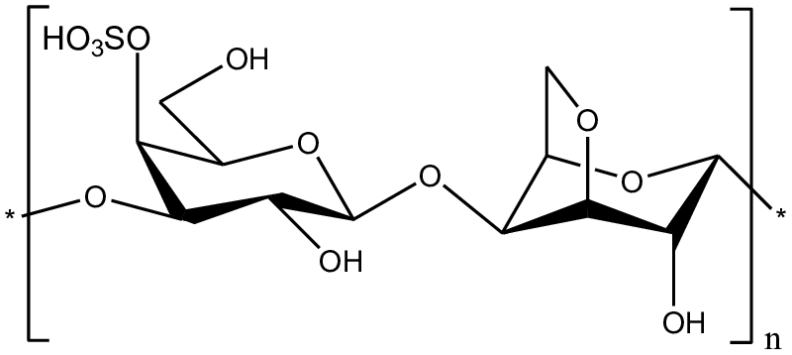


Fig 4.1b. κ-carrageenan Chemical Structure

κ-carrageenan have a linear chain of D-galactopyranosyl units joined with alternating (1 → 3)-a-D- and (1 → 4)-b-D-glycosidic linkages, with most sugar units having one or two sulphate half-ester groups [165]. These sulphate groups are responsible for the negative charge of the polymer, as they are always ionized at the pH values present in foods. There are three major types of carrageenan, kappa (k), iota (i), and lambda (l)-carrageenans [47, 163]. Interestingly, k-carrageenan which has fewer sulfate bonds but is more commonly used in production [166]. Because, of the fewer ester sulfates Kappa can forms strong and rigid gels soluble in hot water. For this reason, we decided to incorporate k-carrageenan (KC) in our experiments. Moreover, (KC) have been predominantly incorporated into more food

matrices based on its ability to form gel under specific conditions and thereby, for also being a vegan friendly alternative to replace gelatin for conventional food systems.

Polymer (κ-carrageenan) properties

κ-carrageenan is a linear polysaccharide with many sulfate groups (pKa ≈ 2.0) providing a strong negative charge across a broad range of pH values [47-48, 163]. However, it can be also found that κ-carrageenan isoelectric pH is around 4.40 in the literature [167]. κ-carrageenan molecular weight ranges from an average of 788.7 g/mol

4.4 Optimization of primary emulsion formation

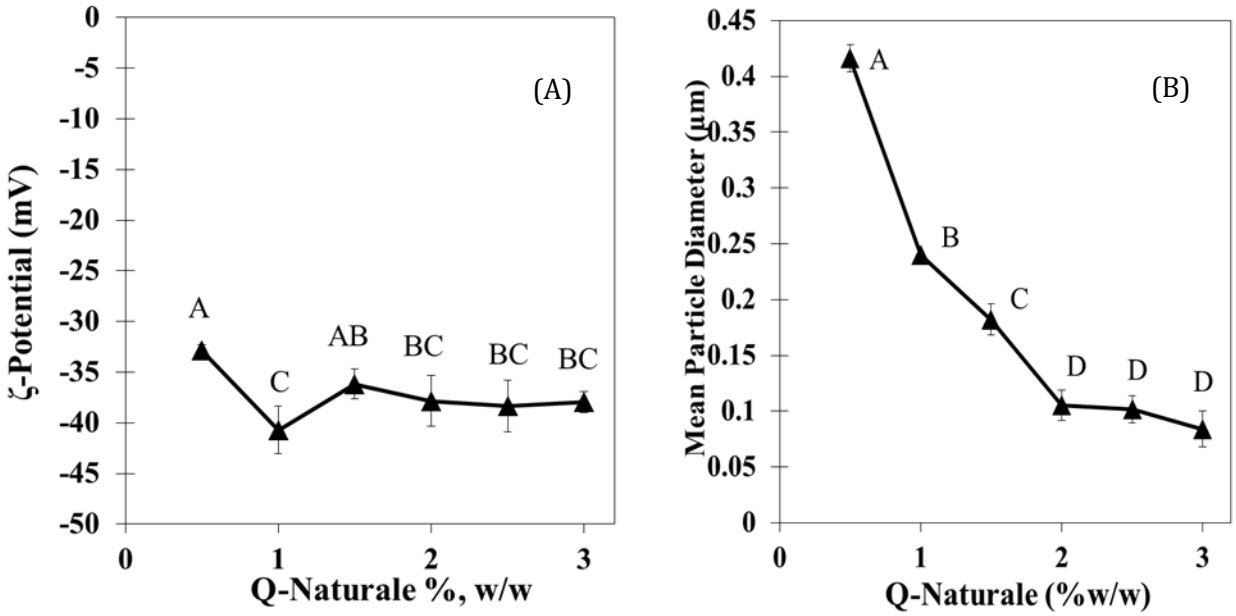


Figure 4.2. Impact of quillaja saponin (Q-Naturale) concentration (%, w/v) on a 10 % fat content of MCT O/W, after homogenization by dual-channel for 3 passes at 12,000 psi

effects on (A) ζ- Potential (B) particle diameter d_{32}. Significant difference was designated with different capital letters (A, B, C).

Initially, the optimum concentration of quillaja saponin required to form the primary emulsions was established using ζ-potential and particle size measurements. Ideally, the emulsifier concentration should be high enough to create small oil droplets during homogenization but not so high that there is a high concentration of free emulsifier in the aqueous phase [161, 168]. If there is too much free emulsifier, then it will interfere with the formation of the next layer by forming electrostatic complexes. The change in the surface potential of the lipid droplets with increasing saponin concentration was measured by microelectrophoresis **(Fig. 4.2a)**. The magnitude of the ζ-potential remained relatively high and constant for all saponin concentrations applied, suggesting that the droplet surfaces were always completely covered by the surfactant. Nevertheless, there was a noticeable decrease in lipid droplet size with increasing saponin concentration (**Fig. 4.2b**), which can be attributed to the fact that a greater surface area of oil can be covered at higher surfactant concentrations. Based on these experiments, a saponin concentration of 1.0% w/v was selected to formulate the primary emulsions as it generated droplets with a high negative charge (-41.6 mV) and low particle diameter (0.24 μm), without having to use a large amount of emulsifier.

Quillaja Saponin (QS) Concentration (%):	Mean Particle Diameter (nm):	ζ- Potential (mV):
0.5	416 ±	-32.88 ± 0.33
1.0	240 ±	-40.7 ± 0.20
1.5	182 ±	-36.16 ± 0.15
2.0	105 ±	-37.83 ± 0.11
2.5	102 ±	-38.32 ± 0.19
3.0	83.9 ±	-37.93 ± 0.08

Table #4.2. Mean Particle Diameter (nm) and ζ- Potential (mV), dependent of Quillaja saponin (QS) concentrations (homogenization by dual-channel for 3 passes at 12,000 psi).

4.5 Optimization of secondary emulsion formation

In our previous study, we found that lipid droplets coated with saponin-PLL layers could be formed at pH 4 [161]. In this series of experiments, our aim was to establish the PLL concentration required to saturate the surfaces of the saponin-coated droplets, without having a high level of free PLL in the surrounding aqueous phase [3, 13, 169]. Any excess cationic PLL might bind to the anionic polysaccharides used to form the tertiary emulsions, thereby interfering with their ability to successfully coat the lipid droplets.

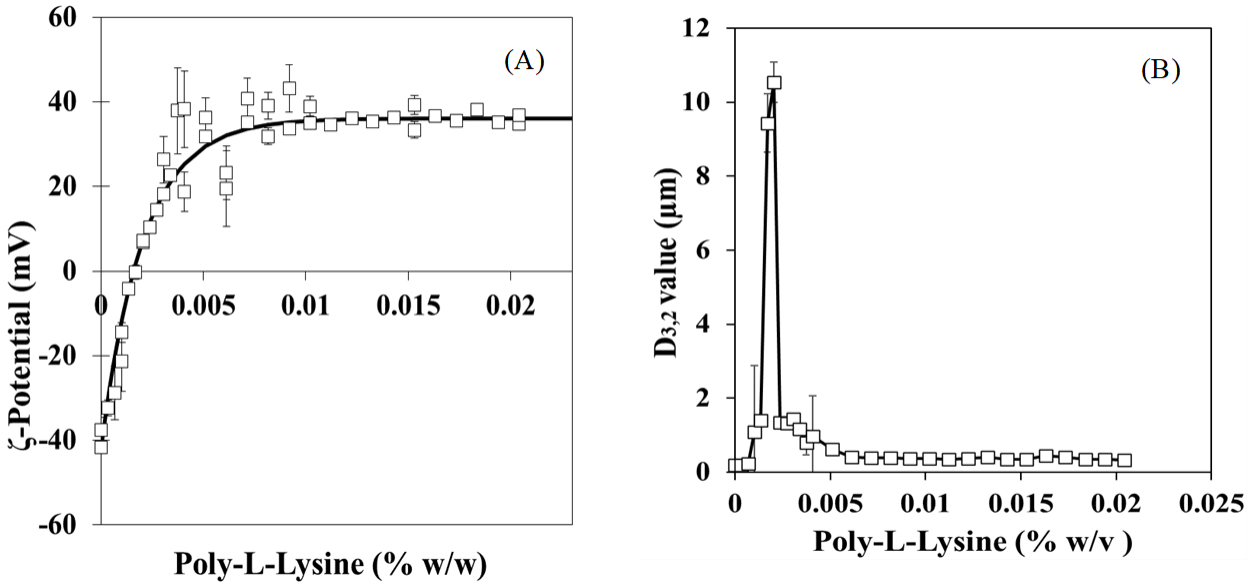


Figure 4.3. Impact of second layer formation with PLL on (A) ζ-Potential and (B) particle diameter (d_{32}) on MCT O/W emulsions containing lipid droplets coated with quillaja saponin (pH 4.0).

As the PLL concentration was increased, the ζ-potential on the lipid droplets went from highly negative (-42 mV) to highly positive (+35 mV), which is consistent with the adsorption of the cationic polypeptides onto the surfaces of the anionic saponin-coated droplets (**Figure 4.3a**). The ζ-potential reached a relatively constant positive value when the PLL concentration exceeded about 0.01%, which suggests that saturation occurred somewhere around this value. A more quantitative analysis was carried out by fitting the following equation to the results [8]:

$$\frac{\zeta(c)-\zeta(\infty)}{\zeta(0)-\zeta(\infty)} = \exp\left(-\frac{c}{3c_{Sat}}\right) \quad (1)$$

Here, $\zeta(c)$, $\zeta(0)$, and $\zeta(\infty)$ are the ζ-potential measurements at a biopolymer concentration of c, in the absence of biopolymer ($c = 0$), and at saturation ($c = c_{Sat}$), respectively. The c_{Sat} value that gave the best fit between the experimental measurements and the theoretical predictions was 0.0062 w/v% (**Figure 4.3a**). This value corresponds to a surface load (Γ) of around 0.10 mg m^{-2}, assuming a disperse phase volume fraction of 0.02 (2% oil droplets) and a particle diameter (d_{32}) of 0.20 μm.

The impact of the PLL concentration on the size of the particles in the secondary emulsions was also measured (**Figure 4.3b**). Ideally, we aimed to form secondary emulsions containing small non-aggregated lipid droplets. Initially, the saponin-coated lipid droplets had a relatively small size (d_{32} = 0.20 μm), because the saponin molecules adsorb rapidly to the droplet surfaces during homogenization and stabilize them against aggregation. When small amounts of the PLL were added, the droplet size remained small, presumably because there was still a high enough negative charge to inhibit droplet flocculation. However, there was a large increase in droplet size at intermediate PLL concentrations, which is attributed to charge neutralization and bridging flocculation. At sufficiently high PLL concentrations, the droplet size decreased again, because the lipid droplets were completely covered by biopolymer and had a strong net positive charge. As a result, there was a strong electrostatic and steric repulsion between the droplets, which inhibited their aggregation.

The overall appearance of the secondary emulsions after storage was consistent with the particle size measurements. The emulsions were stable to creaming at low and high PLL concentrations but underwent rapid creaming at intermediate values. This effect

is attributed to droplet aggregation, which increases the effective particle size, thereby increasing the magnitude of the gravitational forces acting upon the lipid droplets.

Importantly, based on these results, a PLL concentration of 0.01 w/v% was used to form the secondary emulsions in the subsequent experiments. These droplets had a high positive charge (around +40 mV) and a small diameter (around 0.4 μm).

4.6 Optimization of tertiary emulsion formation

Tertiary emulsions were formed by electrostatic deposition of anionic pectin or κ-carrageenan onto the surfaces of the cationic PLL-saponin-coated lipid droplets (pH 4.0). Similar trends were observed for both polysaccharides (**Figure 4.4**). The ζ-potential on the lipid droplets went from highly positive to highly negative as the polysaccharide concentration in both tertiary emulsions was increased (**Figure 4.4a**), indicating that the anionic polysaccharide molecules had adsorbed to the surfaces of the cationic lipid droplets. For both systems, the surfaces of the lipid droplets became saturated around 0.03% w/v polysaccharide. At saturation, the ζ-potential was more strongly negative for the lipid droplets coated by κ-carrageenan (-48 mV) than by pectin (-41 mV), which is attributed to the higher charge density of the carrageenan molecules.

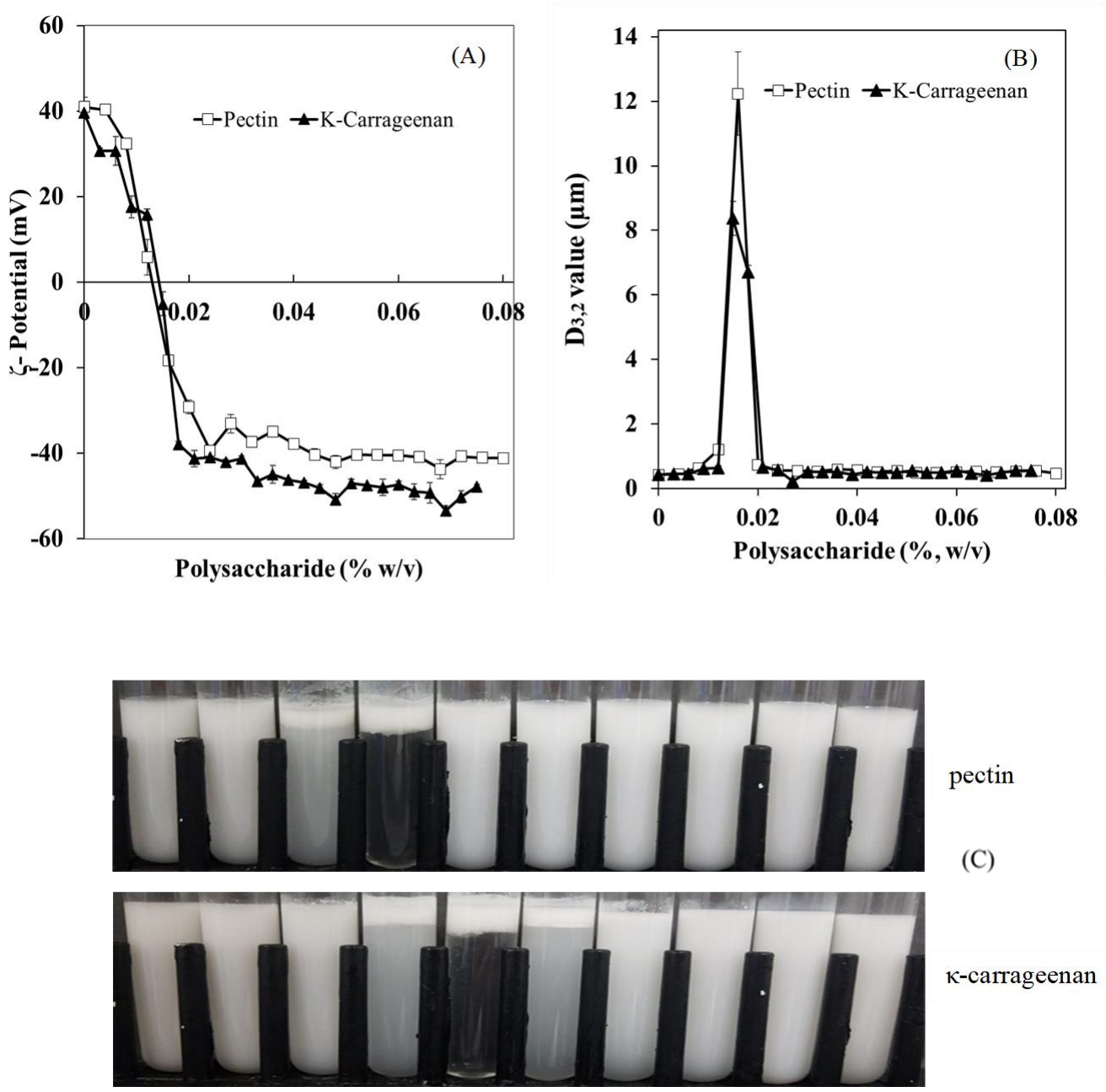


Figure 4.4. Impact of anionic outer layer formation with Polysaccharides % (w/w) (pectin and/or κ-carrageenan) on (A) ζ-Potential (B) mean particle diameter (d_{32}) and (C) appearance of MCT O/W emulsions containing lipid droplets previously coated with PLL (pH 4.0).

The dependence of the particle size on polysaccharide concentration was also similar for both pectin and κ-carrageenan (**Figure 4.4b**). Extensive droplet aggregation was observed at intermediate polysaccharide concentrations, where the net charge was close to zero, which is attributed to charge neutralization and bridging effects. At sufficiently high concentrations, however, relatively small-coated lipid droplets were formed for both polysaccharides (d_{32} = 0.47 μm). The overall appearance of the tertiary emulsions also indicated that they tended to undergo rapid creaming at intermediate polysaccharide concentrations (**Figure 4.4c**), which is consistent with an increase in particle size due to droplet aggregation. Conversely, tertiary emulsions that appeared fairly homogeneous were formed at sufficiently high polysaccharide concentrations, suggesting that they were relatively stable to aggregation and creaming.

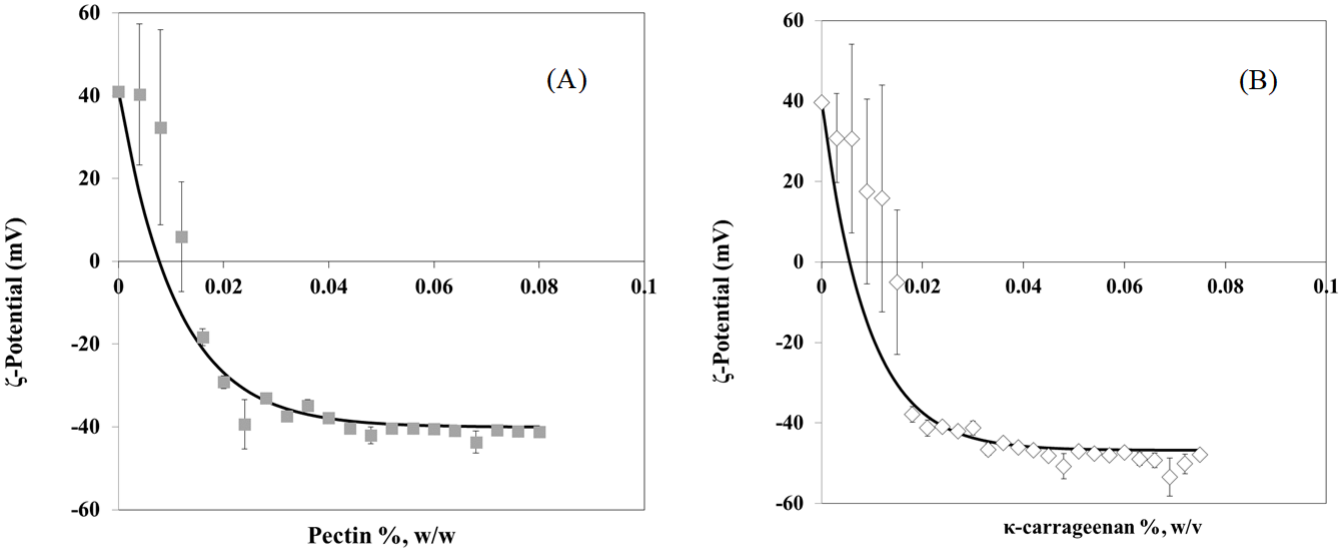


Figure 4.5. Surface ζ- Potential (- mV) Load Calculations from (A) pectin and (B) κ-carrageenan electrostatic depositions over PLL-quillaja saponin secondary emulsions surface droplets.

The surface load of the polysaccharides used to form the outer layers of the tertiary emulsions was calculated using the same model discussed earlier (**Figure 4.5**). For pectin, the c_{Sat} value that gave the best fit between the experimental measurements and the theoretical predictions was 0.00330 w/v% (**Figure 4.5a**), which corresponds to a surface load of around 1.1 mg m^{-2}. For κ-carrageenan, the c_{Sat} value that gave the best fit between the experimental measurements and theoretical predictions was 0.0027 w/v% (**Figure 4.5b**), which corresponds to a surface load of around 0.90 mg m^{-2}. For both calculations, it was assumed that the disperse phase volume fraction was 0.02 (2% oil droplets) and the mean droplet diameter (d_{32}) was 0.40 μm (the measured size of the droplets in the secondary emulsions at saturation). Taken together, these results show that the lipid

droplets can by successfully nano-laminated using food-grade ingredients, such as saponins, polypeptides, and polysaccharides.

4.7 Impact of Environmental Conditions on Multilayers' Stability

In these experiments, the resistance of the primary, secondary, and tertiary emulsions to changes in pH, salt addition, and heating was determined. All the emulsions were diluted to the same final oil level (1.0% w/v) so they could be compared at a similar droplet concentration. The particle size, charge, and microstructure of each emulsion was then analyzed after being subjected to these environmental stresses.

pH-stability

Primary emulsions: For the primary emulsions, the ζ-potential was strongly negative from pH 9 to 4 but was close to zero from pH 3 to 2 (**Figure 4.5a**). This effect is attributed to progressive protonation of the carboxylic acid groups on the adsorbed quillaja saponin molecules when the pH was reduced below their pK_a value, which would be expected to be around pH 3.5 [154]. The mean particle diameter of the primary emulsions was relatively low from pH 9 to 3 but increased appreciably at pH 2 **(Figure 4.5b)**. Moreover, these emulsions were relatively stable to creaming from pH 9 to 3 but exhibited extensive creaming at pH 2 **(Figure 4.5c).** The instability of the emulsions to creaming at the lowest pH is attributed to a reduction in the electrostatic repulsion between the saponin-coated droplets, which should promote droplet aggregation. Droplet aggregation and creaming were also expected to occur at pH 3 because the ζ-potential was also relatively low under these conditions. This suggests that some other factor may also have contributed to the instability of the

emulsions at pH 2. For instance, the ester bond holding the hydrophilic sugar groups to the non-polar regions of the saponin molecules could have been hydrolyzed under acidic conditions [170], thereby reducing the steric repulsion between the droplets.

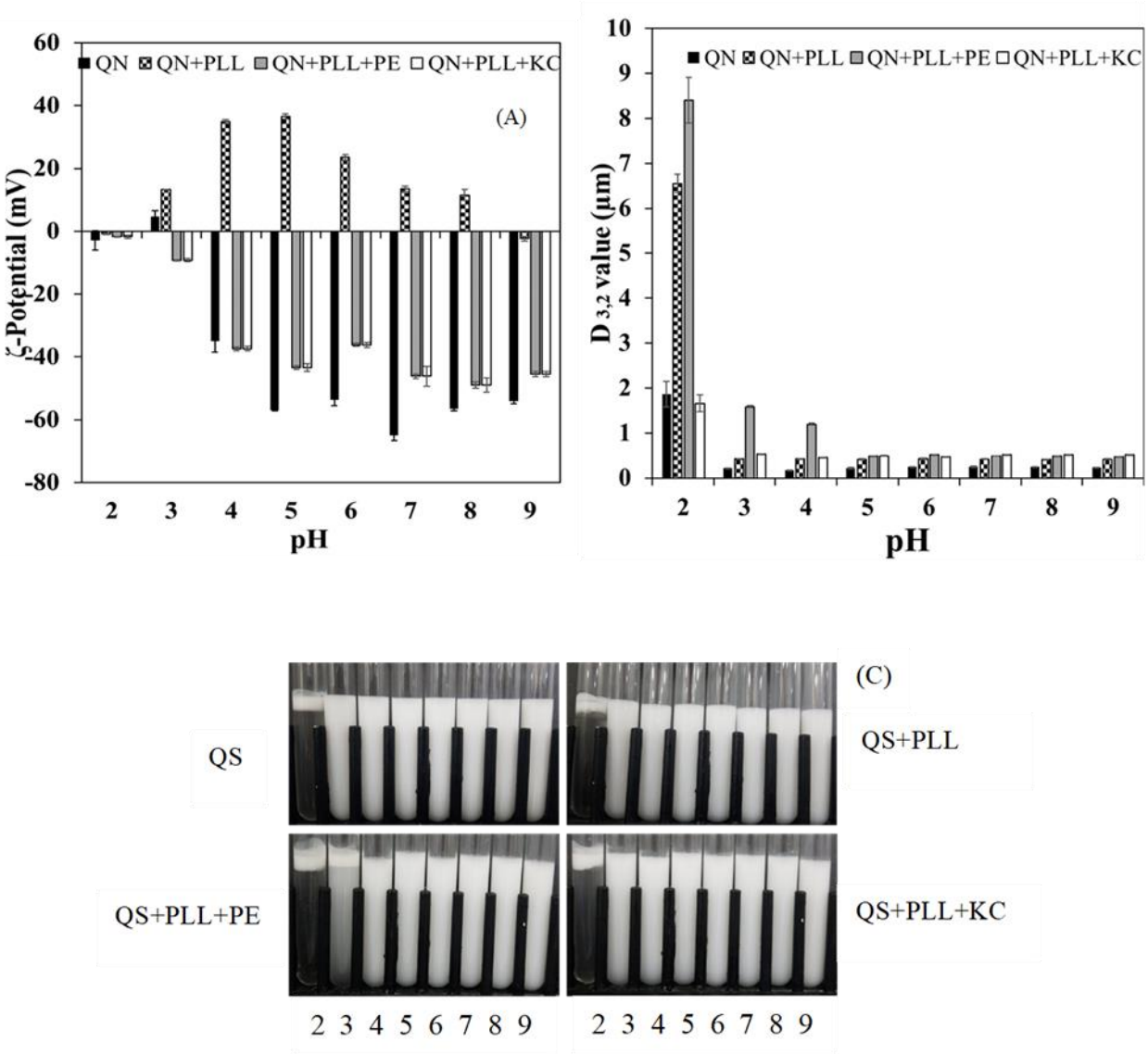


Figure 4.6. pH (2.00-9.00) influence on (A) ζ-Potential, (B) mean particle diameter (d_{32}) and (C) appearance of primary, secondary and both tertiary layers systems, respectively.

Secondary Emulsions: The lipid droplets in the secondary emulsions exhibited very different behavior to those in the primary emulsions when the pH was changed. The ζ-potential of the lipid droplets was strongly positive at pH 4 and 5 but decreased in magnitude as the pH was either lowered or raised. The decrease observed upon lowering the pH is ascribed to the saponin-coated lipid droplets losing their negative charge and so the cationic PLL molecules no longer adsorbed to the droplet surfaces. Conversely, the decrease observed upon increasing the pH is caused by amino groups on the PLL molecules becoming partly deprotonated above their pK_a values and therefore losing their positive charge.

Moreover, the secondary emulsions appeared to be stable to aggregation and creaming from pH 9 to 3 but aggregated appreciably at pH 2. The aggregation observed under the most acidic conditions is attributed to the fact that the PLL molecules had desorbed from the surfaces of the saponin-coated lipid droplets. As a result, they behaved in a similar manner as those in the primary emulsions. The aggregation stability of the lipid droplets in the secondary emulsions at all higher pH values, even though they had very different surface potentials, is probably because the saponin-PLL layer generated a strong steric repulsion between the droplets.

Tertiary Emulsions: The electrical characteristics of the droplets in both tertiary emulsions were similar across the entire pH range. The droplets had a high negative charge

from pH 9 to 4, but a relatively low one from pH 3 to 2 (**Figure 4.6a**). The anionic nature of the droplets at higher pH values are attributed to the outer layer of negatively charged polysaccharide molecules. The reduction in the magnitude of the ζ-potential at lower pH values may have been because the adsorbed polysaccharides lost some of their negative charge and/or because they fully or partially desorbed from the lipid droplet surfaces. The anionic groups on the pectin molecules (carboxyl, $pK_a = 3.5$) would be expected to become protonated at a higher pH than those on the carrageenan molecules (sulfate, $pK_a = 2.0$). However, the charge on the droplets was similar in both tertiary emulsions, suggesting that this effect was not particularly important. Therefore, it seems likely, that the multilayers were fully or partially dissociated from the droplet surfaces under highly acidic conditions.

Despite the similarities in their ζ-pH characteristics, there were appreciable differences in the dependence of the particle size and creaming of the two tertiary emulsions on pH (**Figure 4.6b**). Both emulsions were relatively stable to droplet aggregation at high pH values, which is attributed to the strong electrostatic and steric repulsion between the 3-layer coated droplets. The emulsion with an outer carrageenan layer only aggregated and creamed at pH 2, whereas those with a pectin outer layer aggregated and creamed from pH 2 to 4, indicating that they were less stable under acidic conditions. This may have been because the pectin was attached to the lipid droplets less strongly than the carrageenan, and so promoted some bridging flocculation, *i.e.,* sharing of individual polysaccharide molecules amongst a number of droplets.

Salt-stability

In these experiments, the impact of salt addition on the stability of the lipid droplets in the primary, secondary, and tertiary emulsions was assessed **(Fig. 4.7)**. Our hypothesis was that the presence of the mineral ions would screen any electrostatic interactions in the system. These interactions may have been between the various charged surfactants and biopolymers in the multilayer coatings, as well as between the different lipid droplets. The ionic strength of the aqueous solution surrounding the lipid droplets was increased by adding increasing amounts of salt (0-350 mM NaCl).

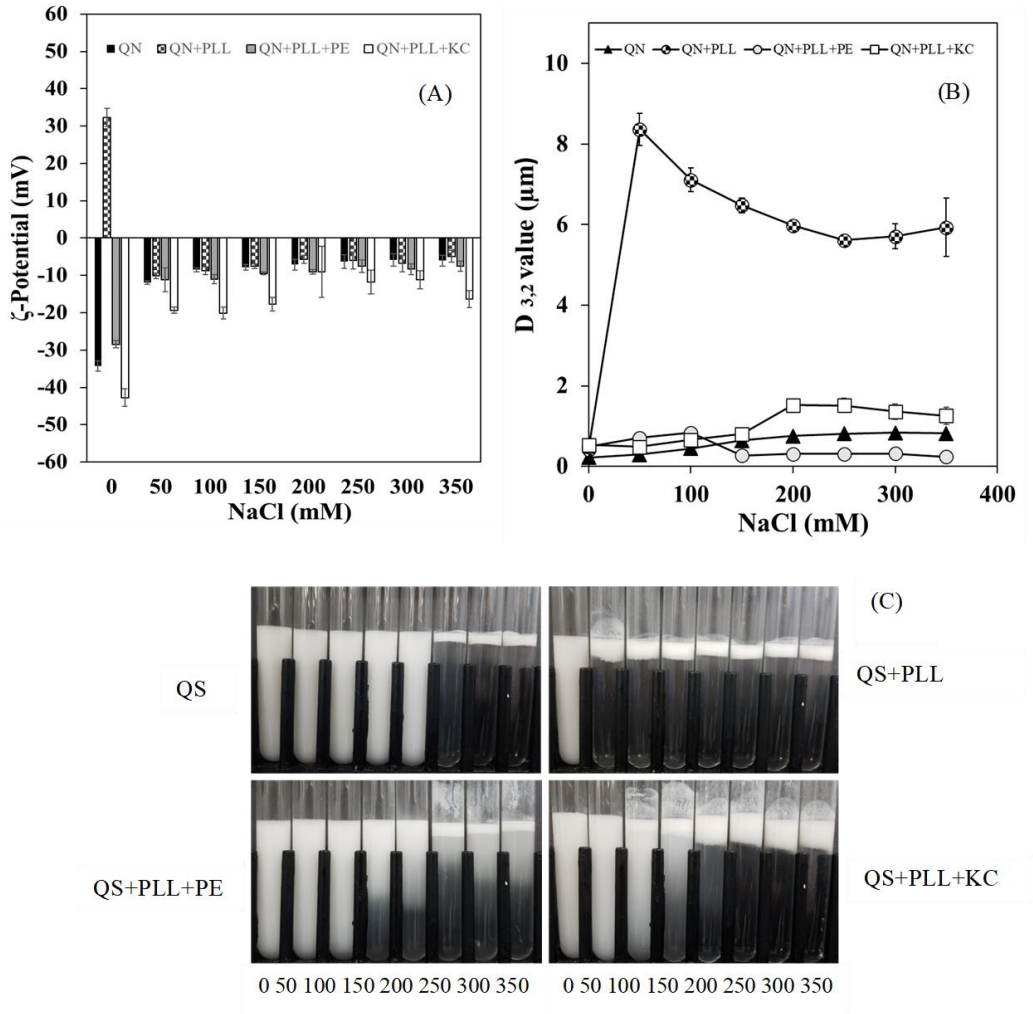


Figure 4.7. (A) ζ-Potential, (B) mean particle diameter (d_{32}) and (C) appearance of primary, secondary and both tertiary layers systems with respective Ionic Strength (0- 350mM) Sodium Chloride interactions.

Primary emulsions: The saponin-coated lipid droplets in the primary emulsions remained negatively charged at all salt concentrations tested **(Figure 4.7a.)**. However, the magnitude of the ζ-potential diminished as the ionic strength was raised due to accumulation of cationic counter-ions (Na^+) around the anionic groups on the lipid droplet surfaces [16]. The size of the particles in the primary emulsions increased slightly as the salt concentration was increased **(Figure 4.7b)**, which is indicative of droplet aggregation promoted by a reduction in the electrostatic repulsion between the lipid droplets. The primary emulsions were relatively stable to creaming up to 200 mM NaCl, but then underwent rapid creaming at higher salt levels (**Figure 4.7c**). This suggests that the droplets may have formed large flocs that creamed rapidly, but that were broken down when the emulsions were diluted for the light scattering experiments.

Secondary emulsions: The ζ-potential of the secondary emulsions went from highly positive in the absence of salt to slightly negative in the presence of any level of added salt, *i.e.,* 50-350 NaCl (**Figure 4.7a**). The charge on the secondary emulsions at these high salt levels was similar to the corresponding primary emulsions. These results suggest that the presence of even low levels of salt were enough to cause the cationic PLL molecules to be displaced from the surfaces of the saponin-coated lipid droplets. In the presence of any level of salt, the secondary emulsions underwent extensive aggregation and rapid creaming

(**Figures 4.7b and 4.7c**). The primary emulsions were relatively stable to creaming from 50 to 200 mM NaCl, whereas the secondary emulsions were highly unstable. This suggests that the PLL molecules may have been weakly attached to the saponin-coated droplets in the secondary emulsions and promoted bridging flocculation. Similar results were obtained in our previous study with a related system [161].

Tertiary emulsions: There was a progressive decrease in the magnitude of the negative ζ-potential on both tertiary emulsions as the salt concentration was increased (**Figure 4.7a**), which is attributed to electrostatic screening effects. In addition, there may have been some desorption of the polysaccharides (and possibly polypeptides) from the lipid droplet surfaces due to the weakening of the electrostatic attraction between the cationic polypeptide and anionic polysaccharide layers. In general, the magnitude of the ζ-potential was higher in the tertiary emulsions containing carrageenan than in the ones containing pectin, which may have been due to the higher charge density of the carrageenan molecules. There were some differences in the impact of salt on the stability of the two tertiary emulsions.

In summary, both emulsions were relatively stable to droplet aggregation and creaming at relatively low salt levels (0 to 100 mM), but exhibited extensive creaming at higher salt levels (**Figures 4.7b and 4.7c**). This was surprising because there appeared to be a decrease in the mean particle diameter in the tertiary emulsions containing pectin at high salt levels (**Figure 4.7b**). This effect may have been because the pectin layer, and possibly also the PLL layer, may have been displaced from the lipid droplet surfaces due to

weakening of the electrostatic attraction between the biopolymer molecules in the multilayer coatings. As a result, there was a decrease in particle size, but the droplets were not stable to aggregation and creaming.

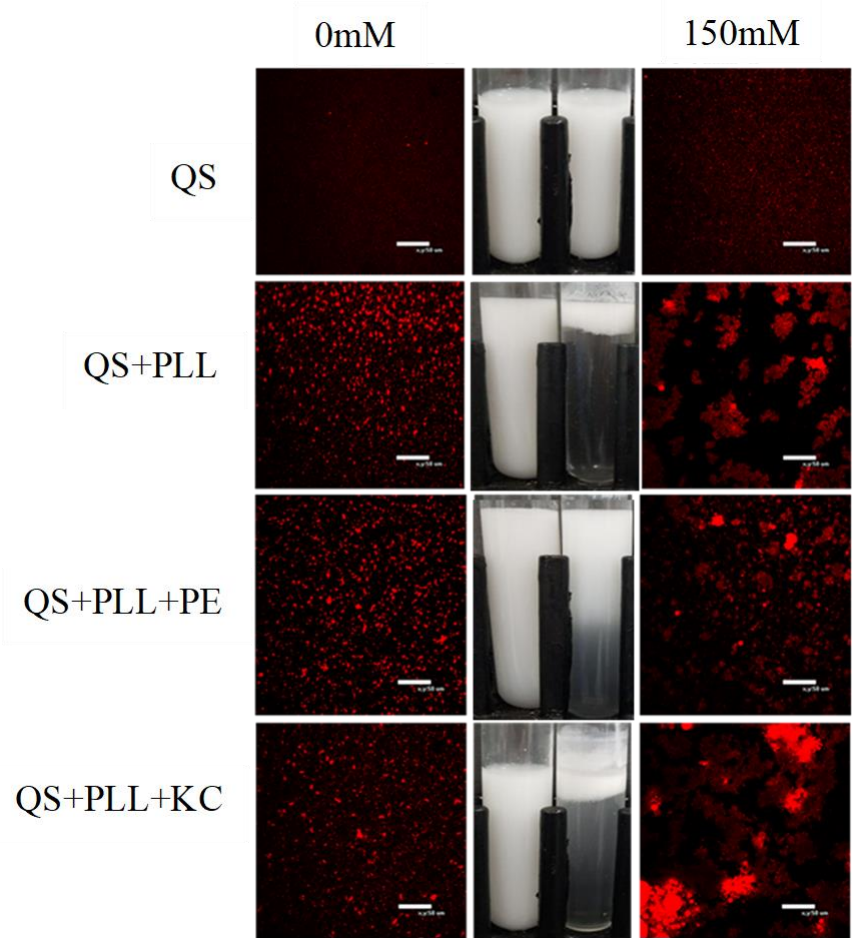


Figure 4.8. Confocal Microscopy images of primary, secondary and both tertiary layers systems with (0 mM and/or 150 mM) sodium chloride interactions.

Confocal microscopy images of the emulsions in the presence of 0 and 150 mM NaCl were also acquired to examine the impact of the interfacial layers on the aggregation stability of the lipid droplets (**Figure 4.8**). These images showed that the primary emulsion was relatively stable to droplet aggregation at this salt level, whereas all of the multilayer emulsions were highly unstable. Overall, these results show that the aggregation and creaming stability of this kind of multilayer emulsion is highly sensitive to salt.

Thermal-stability

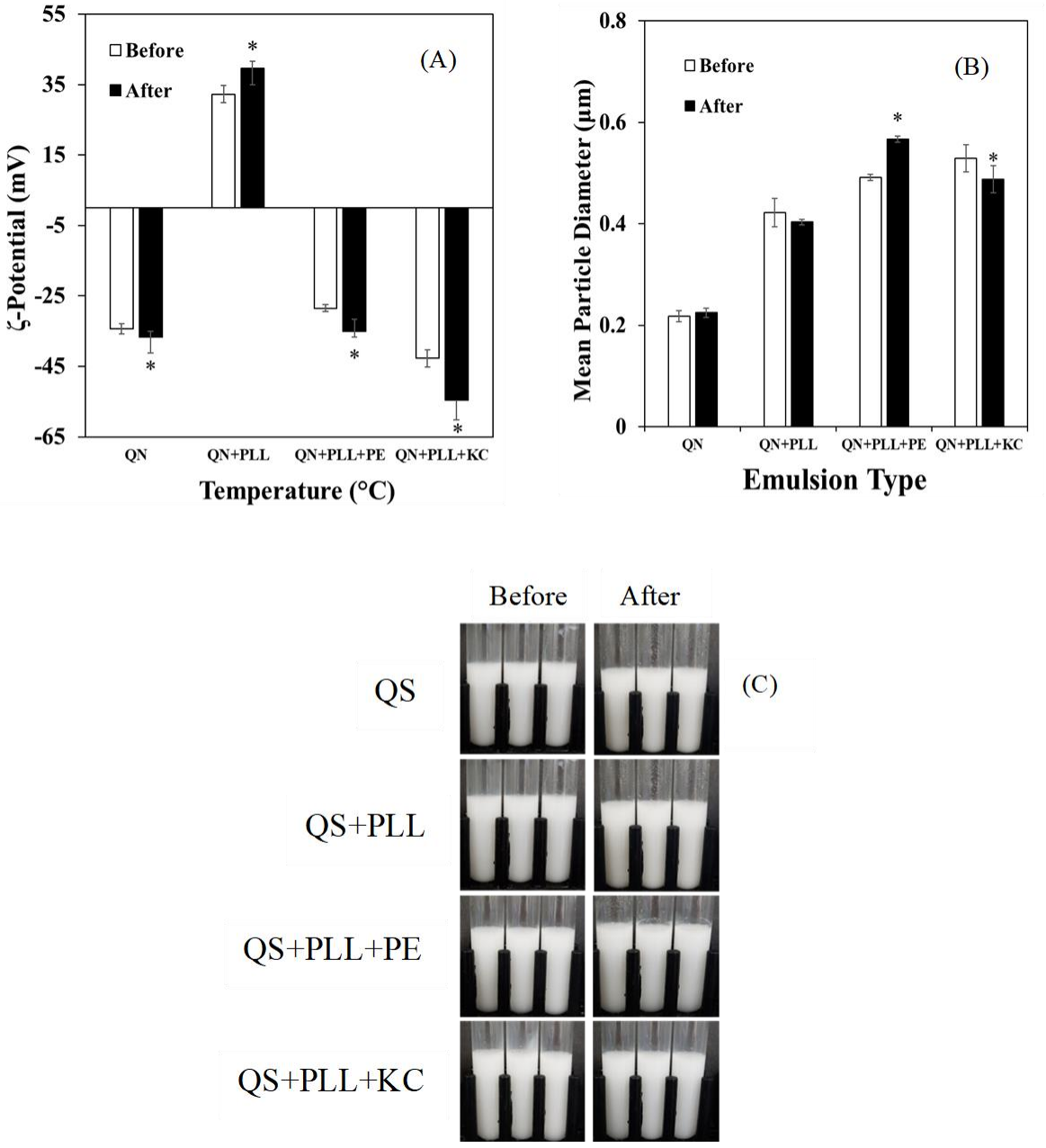


Figure 4.9. "Before vs After" Thermal-Heating exposure effects on (A) ζ-Potential, (B) mean particle diameter (d_{32}) and (C) appearance of primary, secondary and both tertiary layers

systems, respectively. The symbol (*) was used to designate significant difference "Before vs After" treatment.

The thermal stability of the emulsions was determined by measuring their charge, size, and appearance before and after heating at 90 °C for 30 minutes, and then cooling back to ambient temperature **(Figure 4.9)**. These heating conditions were selected to represent thermal conditions that may be used in some food processing operations. In general, it is important to study the precise thermal conditions that a specific food or beverage product experiences, such as pasteurization (*e.g.,* 63°C for 30 minutes or 72 °C for 15 seconds)[171]. The thermal processing conditions used in our study were more severe than typical pasteurization conditions and therefore if the samples were stable to these conditions, then they should also be stable to commercial pasteurization. There was little change in the ζ-potential, mean particle diameter, or appearance of the emulsions after the thermal treatment, which suggested that they all had good heat stability. This phenomenon is attributed to the relatively strong steric and electrostatic repulsion between the droplets under these conditions, *i.e.,* pH 4 and no added salt.

4.7 Conclusions

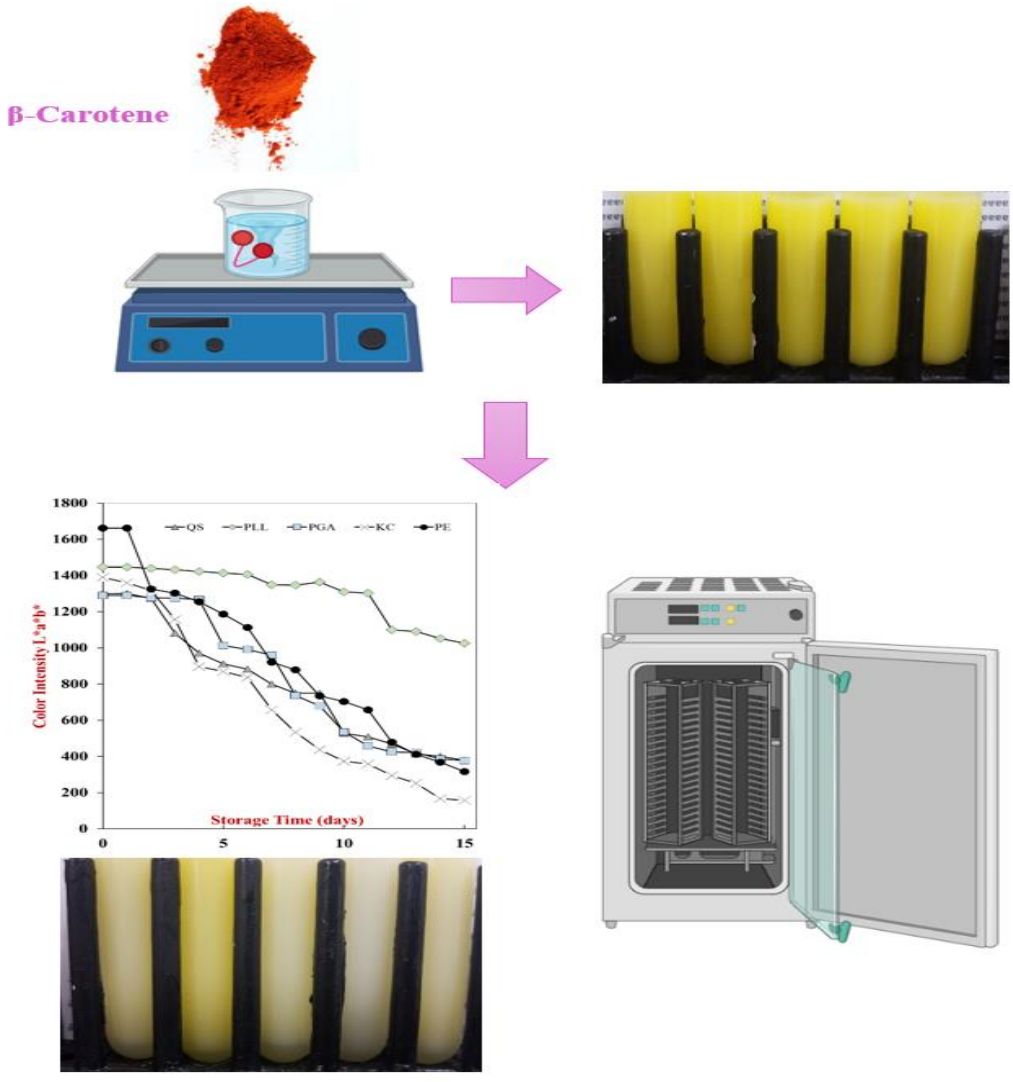
In this study, we showed that multilayer emulsions could be formed by sequential adsorption of anionic emulsifier, cationic polypeptide, and anionic polysaccharide layers onto the surfaces of lipid droplets. Our original hypothesis was, that the multilayer emulsions would have better resistance to environmental stresses than the single-layer emulsions, because they would generate stronger steric and electrostatic repulsion amongst

the lipid droplets. In practice, we found that lipid droplets coated by only quillaja saponin had the best resistance to pH changes and salt addition. This effect was mainly attributed to the fact that acidification or salt addition weakened the electrostatic attraction between the cationic polylysine molecules and the underlying anionic saponin-coated droplets, which promoted droplet flocculation through charge neutralization and bridging flocculation. The presence of an outer polysaccharide layer in the tertiary emulsions improved their stability compared to the secondary emulsions, but they were still not as stable as the primary emulsions. All the emulsions were relatively stable to thermal processing. Overall, our results provide useful insights into the formulation of stable multilayer emulsions from food-grade emulsifiers and biopolymers. There appears to be little advantage to using the multilayer technology to enhance the physical stability of saponin-coated lipid droplets, but there may be advantages in terms of extending their functional properties, which are fully recommended to be explored in future studies.

In summary, our results suggest that multilayer emulsions can be formed from the cationic polypeptide and two anionic polysaccharides used in this study, but that they offer little advantage over single-layer emulsions in terms of their resistance to environmental stresses. In future studies, we intend to determine whether forming these multilayer coatings may have advantages in terms of other functional attributes, such as their ability to inhibit lipid oxidation.

CHAPTER 5. DEGRADATION OF B-CAROTENE IN OIL-IN-WATER NANOEMULSIONS: IMPACT OF NANOLAMINATING DROPLETS WITH SAPONINS, POLYPEPTIDES AND POLYSACCHARIDES

Table of Contents:



Abstract

The kinetics of β-carotene degradation in oil-in-water nanoemulsions was measured. Primary emulsions were formed containing anionic quillaja saponin-coated MCT oil droplets loaded with β-carotene. Secondary emulsions were then formed by depositing cationic polypeptide poly-l-lysine (PLL) onto these anionic droplets. Tertiary emulsions were then formed by depositing anionic poly-glutamic acid (PGA), pectin (PE) or κ-carrageenan (KC) onto these cationic droplets. All the multilayer emulsions were prepared at pH 4.0 to ensure the biopolymers had opposite charges. The kinetics of β-carotene degradation in the different emulsions were then measured when they were incubated at 55°C. In addition, changes in the particle size and ζ-potential of the emulsions were measured using light scattering methods. The chemical stability of the encapsulated carotenoid was highly dependent on the nature the nature of the coating used. The secondary emulsions, which had cationic PLL as an external layer, gave the best protection against color fading, with a final yellowness (b*) of 82% after two weeks. Conversely, the tertiary emulsions, which had anionic polysaccharides or polypeptides as an external layer, gave the worst protection. For instance, when KC was used as the external layer the final yellowness was only 32% after two weeks. These results show that the stability of carotenoids can be improved by controlling the properties of multilayer coatings around oil droplets.

Keywords: multilayers; β-carotene; poly-l-lysine; pectin; poly-glutamic acid; κ-carrageenan.

5.1 Introduction

Carotenoids are one of the most commonly studied hydrophobic nutraceuticals in the food industry [25, 70, 97, 99, 101, 142, 172-176]. Many positive health attributes have been linked to their consumption, including preventing or treating chronic health disorders such as cancer, cardiovascular disease, and macular degeneration [98, 177-180]. In addition, β-carotene exhibits strong provitamin A activity, which is important for individuals who lack this oil-soluble vitamin [180-182]. Most carotenoids have an intense yellow, red, or orange color that increases the aesthetic appeal of foods, and so they are also commonly used as natural pigments in the food industry. There are, however, numerous challenges to utilizing carotenoids as pro-vitamins, nutraceuticals, of pigments in many food products. They are strongly hydrophobic molecules, which means that they typically have to be converted into an emulsified form before they can be introduced into aqueous-based foods and beverages. They are also highly vulnerable to oxidative degradation, which reduces their color intensity and bioactivity. Finally, they typically have a relatively low bioavailability after oral ingestion. Consequently, there is great interest in the creation of emulsified forms of carotenoids using food-grade ingredients and processing operations that can improve their dispersibility, stability, and bioavailability [176, 181]. Nanoemulsions, which contain relatively small (d < 200 nm) emulsifier-coated oil droplets dispersed in water, have been shown to be particularly suitable for this purpose. The functionality of nanoemulsions can be controlled by optimizing the types of oil and emulsifier used to formulate them. However, it can be further extended by using advanced emulsion technologies, such as forming multilayer coatings around the oil droplets.

In this study, we focused on the utilization of the multilayer coating technology to improve the chemical stability of β-carotene encapsulated within oil-in-water

nanoemulsions. Multilayer nanoemulsions are normally formed through the sequential electrostatic deposition of charged biopolymers (proteins or polysaccharides) onto the surfaces of oppositely charged oil droplets [51, 54, 81]. This process can be repeated a number of times to add extra layers to the coating [76, 181]. Coating oil droplets with multilayers can lead to the creation of food emulsions with improved resistance to environmental stresses like pH changes, salt addition, heating, freezing, or dehydration [3]. Moreover, multilayer coatings can be designed to enhance the chemical stability of encapsulated components, as well as to modulate their release within the gastrointestinal tract. The functional attributes of multilayer emulsions can be tuned for specific applications by varying the type, number, and sequence of the biopolymers used to coat the oil droplets [150].

For this reason, we explored the possibility of producing multilayer nanoemulsions that could be used to improve the dispersibility and stability of β-carotene. Previously, we showed that anionic saponin-coated oil droplets could be coated by a layer of cationic poly-L-lysine (PLL) to form secondary nanoemulsions[19]. In addition, we showed that these droplets could be further coated with cationic polypeptides such as polyglutamic acid (PGA), or with polysaccharides such as pectin (PE) or κ-carrageenan (KC), to form tertiary nanoemulsions [19-20]. Previous research has shown that the rate of β-carotene degradation increases with decreasing pH and increasing temperature[97, 99]. For this reason, we performed accelerated stability studies by incubating the β-carotene-loaded multilayer nanoemulsions at pH 4.0 and 55 ºC for 15 days. Therefore, we hypothesized that by engineering the surface emulsion membrane with different layer compositions (surfactants, polypeptides, linear and branched dietary fibers) we will indeed acquire a broader knowledge in how to modulate β-carotene degradation rates.

5.2. Materials and Methods

5.2.1. Materials

A medium chain triglyceride oil (Miglyol 812 N) was purchased from IOI Oleo GmbH (Witten, Germany). Quillaja saponin (Q-Naturale™ 200) was provided by National Starch LLC (Bridgewater, N.J.). The cationic polypeptide epsilon-poly-L-lysine (MW = 2.5 to 3.8 kDa) (CAS No. 73548-20-60) and anionic poly-glutamic acid (MW ≥ 700 kDa) (CAS No. 25513-46-6) were purchased from Wilshire Technologies Inc. (Princeton, NJ, USA). β-carotene (CAS No. 7235-40-7) and anionic polysaccharides were purchased from the Sigma Chemical Company (St. Louis, MO): pectin (from citrus peel) and κ-carrageenan (sulfated plant polysaccharide). All other chemicals were of analytical grade. Double distilled water obtained from a commercial water purification system (NANOpure Infinity, Barnstead International, Dubuque, IA) was used for the preparation of all solutions and emulsions.

5.2.2. Preparation and characterization of primary nanoemulsions

Carotenoid-fortified primary nanoemulsions were fabricated and characterized according to the methods described previously[173, 179, 183-184]. Briefly, oil phases were prepared by dispersing β-carotene (0.1 wt%) in medium chain triglyceride oil held at 50 ºC, followed by sonicating and stirring until total dissolution was observed. Aqueous phases were prepared by dispersing quillaja saponin (1.0 wt%) in double distilled water at pH 4.0. A saponin concentration of 0.1% w/v was used to prepare the nanoemulsions based on the results of our earlier studies, as this concentration gave small highly charged droplets with little free emulsifier being present in the surrounding aqueous phase [20]. The oil and water phases were combined using a high-speed blender (10,000 rpm, 2 min) and then sonicated during 10 minutes (Sonicator FB505, Thermo Fisher Scientific, Waltham, MA, USA). These

coarse emulsions were then passed through a microfluidizer (12,000 psi, 3 passes) to reduce the droplet size further (M110Y, Microfluidics, Newton, MA).

5.2.3. Preparation of biopolymer coating solutions

As described previously [20], epsilon-poly-L-lysine (PLL) solutions were prepared by dispersing 0.1% w/v of the powdered ingredient in double distilled water and stirring continuously for at least 2 h at ambient temperature to ensure dissolution. Similarly, polyglutamic acid (PGA) and pectin (PE) solutions (0.3 w/v %) were prepared by dispersing the powdered ingredients into double distilled water and stirring for at least 2 h. The κ-carrageenan (KC) solutions were prepared by dispersing powdered carrageenan (0.3%, w/v) in distilled water and then heating at 70°C for 20 min, cooling to room temperature and the stirring for 1 h to ensure dissolution. All the solutions were then adjusted to pH 4.0 using NaOH and/or HCl.

5.2.4. Preparation of secondary and tertiary nanoemulsions

Both secondary and tertiary emulsion layers were prepared using a method described in our previous article [161], and so only a brief description is given here with any modifications highlighted.

Secondary nanoemulsions: The secondary nanoemulsions were prepared by mixing 2 mL of primary nanoemulsion, 1 mL of PLL (0.1 w/w %) solution, and 7 mL of double distilled water, all at pH 4.0. The final secondary nanoemulsions therefore contained 0.01% PLL. This concentration was used because previous experiments showed that it was sufficient to completely coat the surfaces of the oil droplets without leading to excess PLL in

the surrounding aqueous phase (which would interfere with the subsequent formation of the tertiary layers) [19-20]. These nanoemulsions were then stored for 24 h to allow them to come to steady state.

Tertiary nanoemulsions: The tertiary nanoemulsions were formed by mixing 5 mL of secondary nanoemulsion, 1 mL of anionic biopolymer solution (0.3% w/w PGA, KC, or PE), and 4 mL of double distilled water, all at pH 4.0. The final tertiary nanoemulsions therefore had a final anionic biopolymer concentration of 0.03%. These nanoemulsions were then incubated at ambient temperature for 24 h to allow them to reach steady state.

After formation the primary, secondary and tertiary emulsions were all adjusted to the same final oil concentration (1.0 % w/w) and pH (4.0). Then, the samples were covered with aluminum foil to block light and placed in a 55°C incubator to accelerate carotenoid degradation. Samples were then periodically removed and analyzed throughout the 15 day storage period.

5.2.5. Characterization of physicochemical properties

5.2.5.1. Extraction and analysis of β-carotene

The β-carotene was extracted from the samples and analyzed according to a method previously described (Yuan, Gao, Zhao, & Mao, 2008) with slight modifications. Briefly, an organic solvent consisting of 2:3 (v/v) hexane/isopropanol was used to extract the carotenoids. The samples were then shaken to intimately mix the solvent and nanoemulsion. The resulting mixtures were then allowed to separate into two phases and the organic phase containing the carotenoids was removed. The β-carotene concentration in the organic phase was then measured at 450 nm using a UV-visible spectrophotometer,

which was blanked using 2:3 (v/v) hexane/isopropanol solution[101, 185]. The β-carotene concentration was then determined from a standard curve that had been prepared with solutions of known concentration.

5.2.5.2. Color degradation analysis

Periodic analysis of the tristimulus color coordinates of the carotenoid-loaded nanoemulsions was performed using a colorimeter (ColorFlex EZ 45/0-LAV, Hunter Associates Laboratory Inc., Virginia, USA). The colorimeter was first calibrated with white and black standard backplates and then the L* (lightness), a* (redness/greenness) and b* (yellowness/blueness) parameters of the samples were measured throughout storage. The total difference in the color value (ΔE*) was then calculated:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}\,. \qquad (1)$$

Here, ΔL*, Δa* and Δb* are the differences between the color coordinates measured after a certain storage time and the initial values.

5.2.5.3. Particle dimensions and particle surface charges

The particle size characteristics of the nanoemulsions were measured by laser diffraction (Mastersizer 3000, Malvern Instruments, Worcestershire, United Kingdom), while the particle charge characteristics (ζ-potential) were measured by microelectrophoresis (Nano-ZS, Malvern Instruments). All samples were diluted prior to

analysis with double distilled water adjusted to pH 4.0 to obtain a sufficiently strong light scattering signal for analysis, while avoiding multiple scattering effects.

5.2.5.4. Storage stability studies

The stability of each β-carotene loaded layer-by-layer nanoemulsions were studied in terms of particle size. In terms of storage stability effects on particle size, we used this simple equation:

$$\%SizeIncrease = 100X\frac{d_{15} - d_0}{d_0}. \quad (2)$$

The stability of each β-carotene loaded layer-by-layer nanoemulsions were also studied, but in terms of surface charge (ζ-potential). Similarly, as the particle size measurements experiments, a simple equation was used for the sake of comparison from day 0 to day 15 on surface charge (ζ-potential).

$$\%ChargeDifferential = 100X\frac{\zeta d_{15} - \zeta d_0}{\zeta d_0}. \quad (3)$$

The microstructures of the lipid particles and oil bodies in each β-carotene loaded layer-by-layer nanoemulsions were observed by confocal fluorescence microscopy. Briefly, 200 μL samples were dyed by adding 20 μL fat-soluble stain (1 mg/mL Nile Red in ethanol). Then, 5 μL samples were placed on a glass microscope slide and covered by a glass cover slip before being observed under an 80× objective lens (Nikon D-Eclipse C1 80i, Nikon, NY, United States).

Statistical analysis

All experiments were performed in triplicate and the means and standard deviations were calculated.

5.3 Results and Discussion

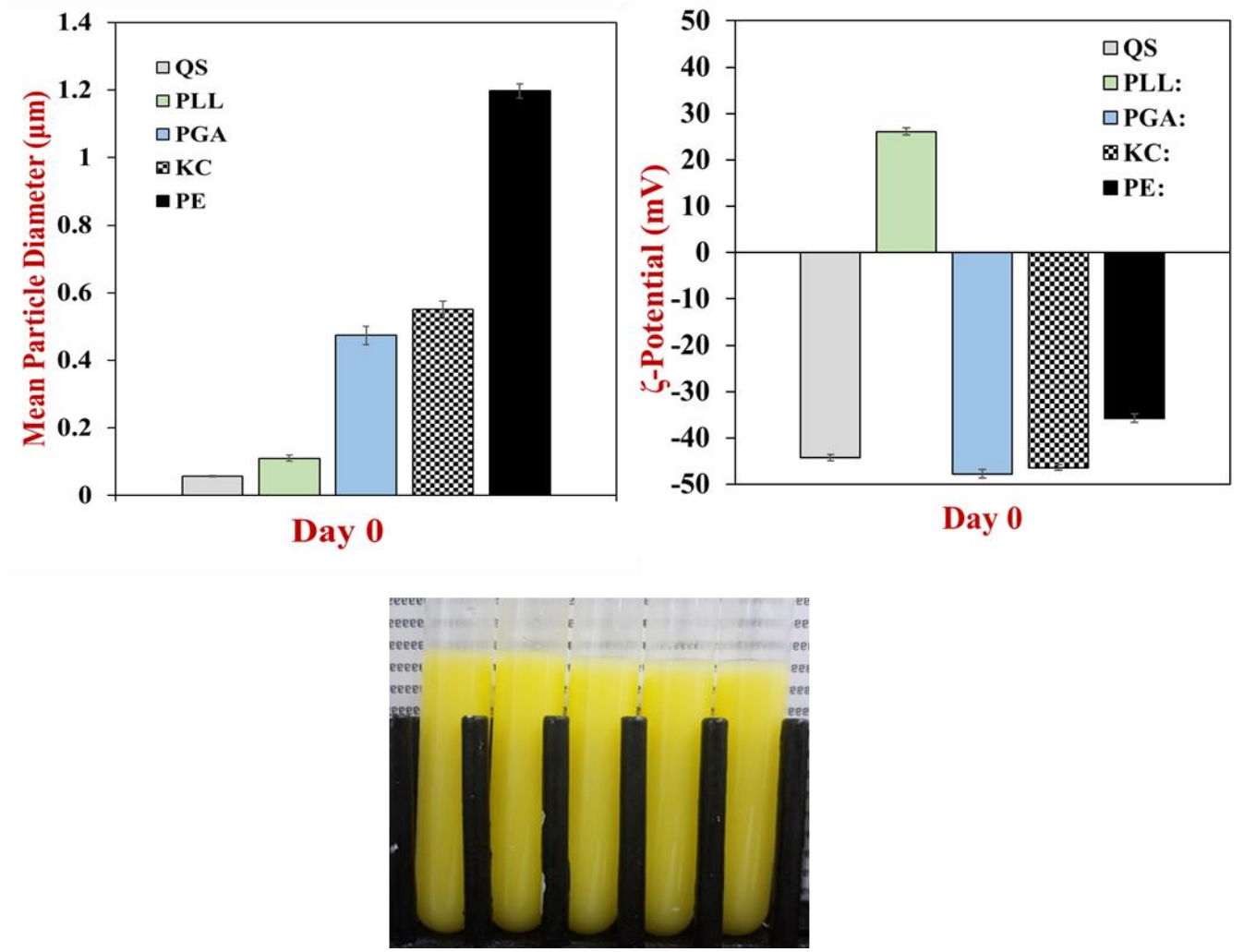


Figure 5.1: (A) Mean particle diameter (μm), (B) Surface ζ-potential (mV), and (C) visual appearances of: β-carotene-loaded primary, secondary, and tertiary emulsions at pH 4.0 (day 0).

5.3.1. Formation of β-carotene-loaded primary, secondary and tertiary nanoemulsions

The droplet charge and size characteristics of the primary, secondary, and tertiary nanoemulsions were measured, and their overall appearance was recorded (**Fig 5.1**). The ζ-potential of the primary nanoemulsions was strongly negative (-44.2 mV) due to the adsorption of anionic quillaja saponin molecules to the oil droplet surfaces during homogenization. As expected, the ζ-potential of the secondary nanoemulsions was positive (+26.2 mV) as a result of adsorption of cationic PLL molecules around the anionic saponin-coated oil droplets. Finally, the ζ-potential values of the tertiary nanoemulsions were all negative (-47.3, -46.4, and -35 mV) because the anionic biopolymers (PGA, KC, or PE) adsorbed on top of the cationic PLL layer. The observed difference in the magnitudes of the ζ-potentials on the droplets in the tertiary nanoemulsions can be attributed to differences in the electrical characteristics of the anionic biopolymers used to form the outer coating. PGA and pectin have carboxylic acid groups ($pK_a \approx 3.5$) that should be partially charged at pH 4. PGA has a higher linear charge density than pectin, since it has carboxylic acid groups attached to every amino acid unit, which would account for the higher ζ-potential of the tertiary nanoemulsions formed from PGA. The κ-carrageenan has one sulfate group ($pK_a \approx 2$) per disaccharide unit and would therefore also be expected to have a high net charge at pH 4.

The mean particle diameter was relatively small (d_{32} = 0.058 μm) in the primary nanoemulsions because quillaja saponin is an effective emulsifier and microfluidization is an effective homogenization method. There was an appreciable increase in mean particle diameter (d_{32} = 0.110 μm) after PLL was added to the primary nanoemulsions, which may

be due to the increase in interfacial thickness and/or some droplet flocculation. There was a much more substantial increase in the mean particle diameter (d_{32} = 0.474, 0.551, and 1.19 μm) after PGA, KC or PE were added to the secondary nanoemulsions, which suggests that appreciable droplet aggregation occurred in the tertiary systems. The PE-tertiary nanoemulsions appeared to have the greatest degree of droplet aggregation, which may have been because they had the lowest surface charge, so there was a weaker electrostatic repulsion between them. Even so, all of the nanoemulsions initially had a uniform yellowish appearance, which was attributed to selective light absorption by the β-carotene and light scattering by the nanoemulsion droplets.

5.3.2. Impact of storage on nanoemulsion properties:

5.3.2.1. Color fading

The whiteness (**L***), redness/greenness (**a***) and yellowness/blueness (**b***) parameters of the primary, secondary, and tertiary nanoemulsions were measured throughout storage using a colorimeter. The total color difference (ΔE) of each sample was then calculated from these values as described earlier (**Fig. 5.2a**). Photographs of the samples were also taken before and after storage for 15 days (**Fig. 5.2b**). The yellowness (positive b* value) of the samples before and after storage was also reported (**Fig 5.3c.**).

The initial cloudy yellow appearance of all the nanoemulsions can be attributed to light scattering by the oil droplets and selective absorption of light waves by the carotenoids and saponins [15, 154, 186]. During there was strong evidence of color fading in all of the nanoemulsions, which can be attributed to the fact that the elevated temperature and

high acidity of the samples promoted carotenoid degradation. However, there were differences in the kinetics of β-carotene loss depending on the formulation (**Fig. 5.2a**).

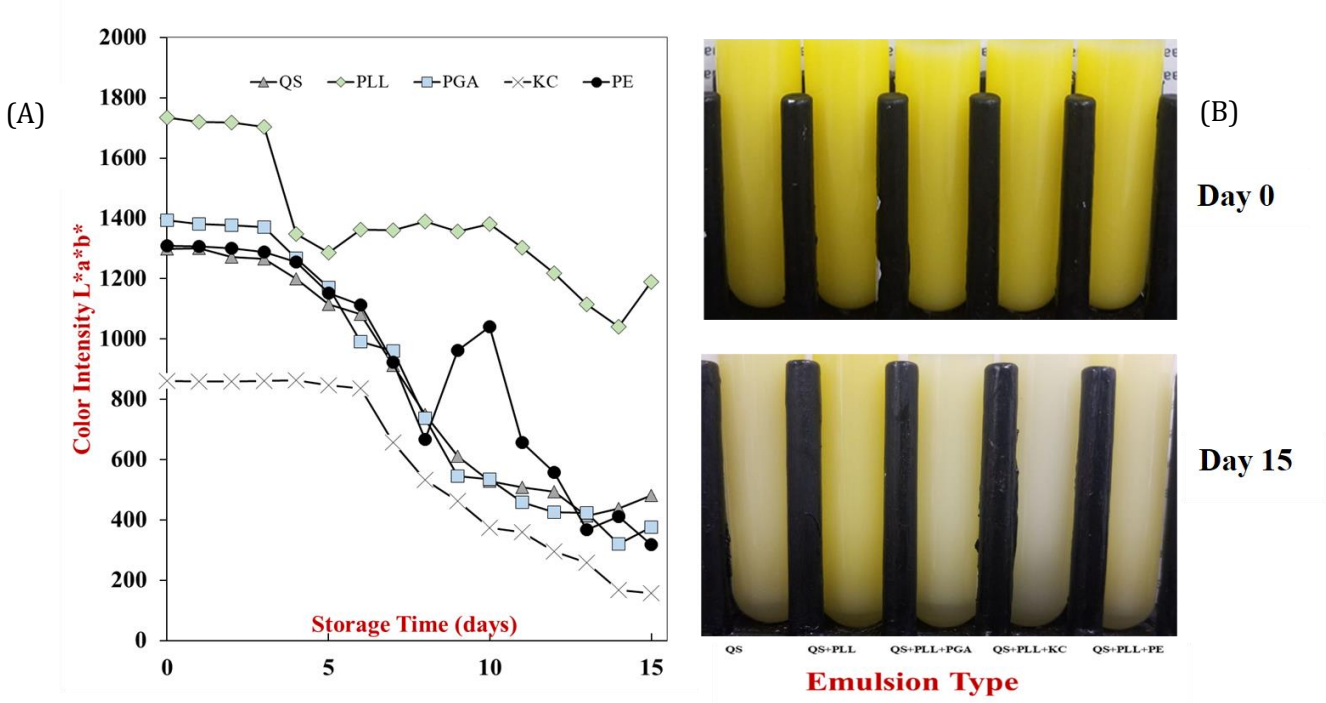


Figure 5.2. (A) Colorimeter results, (B) Appearances of (QS, PLL, PGA, KC, PE) β-Carotene-loaded layers systems at pH 4.0 "before" and "after" being storage at 55 °C for 15 days.

The optical properties of the samples remained fairly constant for the first few days of storage (**Fig. 5.2.a**), which may have been due to the presence of some natural antioxidants that were preferentially oxidized, such as the saponins or proteins. Presumably, after this time these antioxidants were expended, and so the β-carotene then oxidized leading to color fading. Interestingly, the rate of color fading was less in the secondary nanoemulsions that had a cationic outer coating than in the primary and tertiary nanoemulsions that had anionic outer coatings. This effect may have been because oil

droplets with a positive charge can electrostatically repel transition metal ions (such as Fe^{2+} and Fe^{3+}) from their surfaces, thereby preventing them from coming into close contact with the encapsulated carotenoids. Ferric and ferrous ions are known to be potent pro-oxidants that can accelerate the chemical degradation of carotenoids. The ability of the cationic secondary layers to reduce color fading can be seen in the photographs of the samples after 15 days storage (**Fig. 5.2b**). These samples still had a slightly yellow tinge after storage, whereas all the other samples were almost white. In addition, the reduction in yellowness of the secondary nanoemulsions throughout storage occurred more slowly than in the other nanoemulsions (**Fig. 5.2c**). Taken together, these results show that the oil droplets coated by a layer of saponin and PLL were the most resistant to color fading during storage.

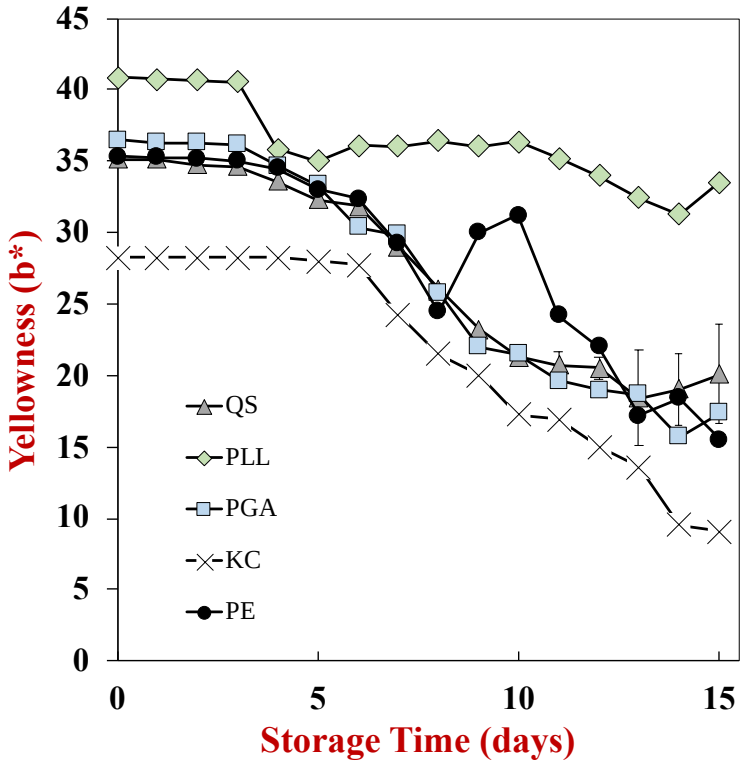


Figure 5.3. Change in the yellowness (b*) of the primary, secondary, and tertiary nanoemulsions during storage. The nanoemulsions are designated by their outer layers.

5.3.2.2. β-carotene degradation

The origin of the color fading in the nanoemulsions was attributed to the degradation of the β-carotene during storage. To confirm this hypothesis, the decrease in the curcumin concentration over time was measured (**Fig. 5.4.**). These measurements clearly showed that there was a reduction in the amount of β-carotene in all of the nanoemulsions with time, which supports this hypothesis. Nevertheless, there were differences in the rate of β-carotene degradation in the different nanoemulsions.

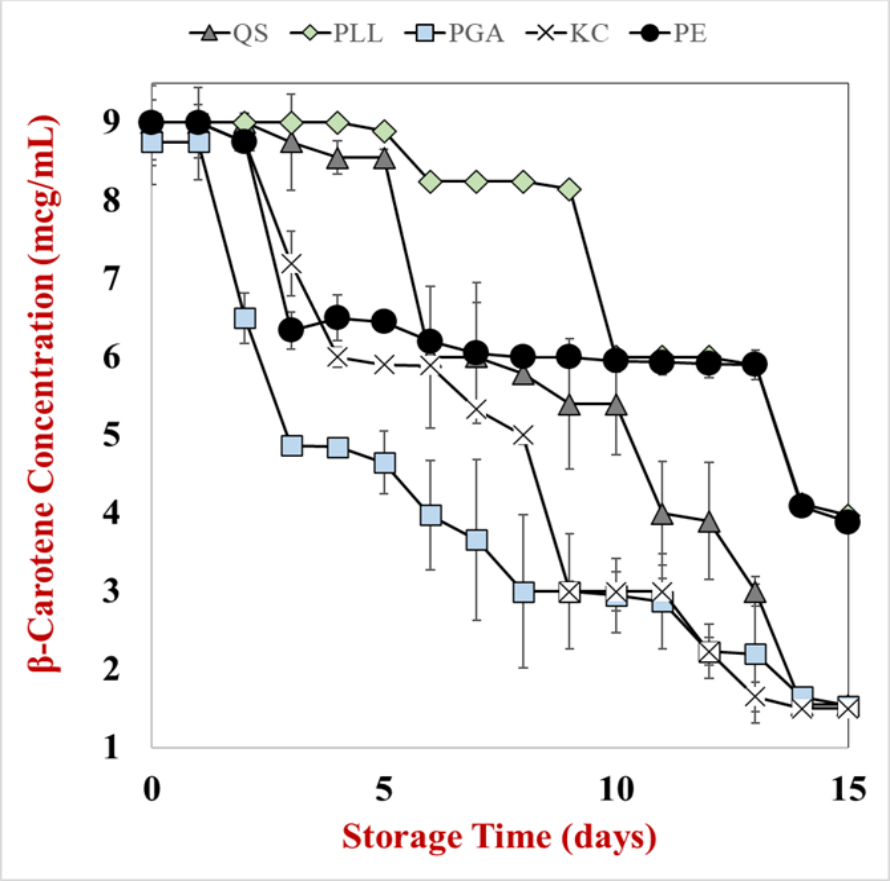


Figure 5.4. Change in β-carotene concentration (mcg/mL) in the primary, secondary, and tertiary nanoemulsions during storage at 55°C (pH 4).

As observed in the color fading experiments, there appeared to be a lag period of a few days when the β-carotene concentration remained relatively constant. Again, this may because there are some antioxidants in the nanoemulsions that inhibited oxidation in the early stages but became depleted at later times. The rate of β-carotene degradation was considerably less in the cationic secondary nanoemulsions than then anionic primary and tertiary nanoemulsions, which is consistent with the color fading experiments. As discussed

earlier, this may have been because cationic coatings could repel cationic transition metal ions away from the oil droplet surfaces, thereby protecting the encapsulated carotenoids from iron-catalyzed degradation. Previous studies have also shown that oil droplets coated with cationic biopolymers have improved resistance to lipid oxidation, *e.g.,* salmon oil droplets coated by whey protein under acidic conditions [55, 62, 84, 189].

Table #5.1. Mean particle diameter and ζ-potential values of β-carotene-loaded multilayer nanoemulsions before and after storage at 55°C (pH 4) for 15 days.

Outer Layer	**Mean Particle Diameter (μm)**		**ζ-potential (mV)**	
	Before	After	Before	After
QS	0.058 ± 0.003	0.066 ± 0.002	-44.2 ± 0.10	-42.55 ± 0.30
PLL	0.110 ± 0.004	0.112 ± 0.001	+26.15 ± 0.23	+31.25 ± 0.11
PGA	0.474 ± 0.002	0.507 ± 0.003	-47.73 ± 0.14	-45.62± 0.30
KC	0.551 ± 0.001	0.561 ± 0.001	-46.37 ± 0.29	-39.47 ± 0.36
PE	1.195 ± 0.001	1.203 ± 0.001	-35.75 ± 0.09	-36.35 ± 0.11

5.3.2.3. Physical stability of nanoemulsions

For commercial applications, it is important that nanoemulsion-based delivery systems remain physically stable throughout storage. For this reason, we also measured the change in the size and charge of the particles in the different nanoemulsions over time (**Table 5.1**). For all the nanoemulsions, there was a small increase in the mean particle diameter after storage, but this increase was relatively modest (< 15%). This suggested that all the nanoemulsions were relatively stable to aggregation during storage, even when held at an elevated storage temperature. This effect can probably be attributed to the strong steric and electrostatic repulsive forces associated with the biopolymer coatings, which prevented them from coming close together. There was also little change in the electrical characteristics of the droplets during storage. All the droplets in the primary and tertiary nanoemulsions remained strongly negative, while those in the secondary nanoemulsions remained strongly positive. There was, however, a modest increase in the magnitude of the positive charge in the secondary nanoemulsions after storage, which may have been due to slow adsorption of any PLL molecules remaining in the aqueous phase or to rearrangement of the adsorbed PLL molecules at the droplet surfaces over time.

Changes in the microstructure of the different nanoemulsions after storage were obtained using confocal fluorescence microscopy (**Figure 5.5**). Before storage, all of the nanoemulsions contained small oil droplets (stained red) that were dispersed throughout the water phase (black). After storage, there was evidence of droplet aggregation in some of the nanoemulsions, with an increase in the dimensions of the lipid-rich domains. In the primary nanoemulsions, there were a few large irregular-shaped lipid-rich particles present after storage, which suggests that some flocculation had occurred. These flocs may not have

been observed in the light scattering measurements because they dissociated after dilution and stirring, which was part of the sample preparation procedure for the laser diffraction measurements. Similarly, there appeared to be numerous flocs in the tertiary emulsions with an outer PGA coating after storage, again suggesting that some droplet aggregation had occurred. Once possible explanation of this phenomenon in bridging flocculation, *i.e.,* part of the PGA molecules became partially detached from one oil droplet and then attached to another oil droplet, thereby linking them together. Again, these may have been weak flocs that were dissociated when the samples were diluted for analysis in the light scattering device.

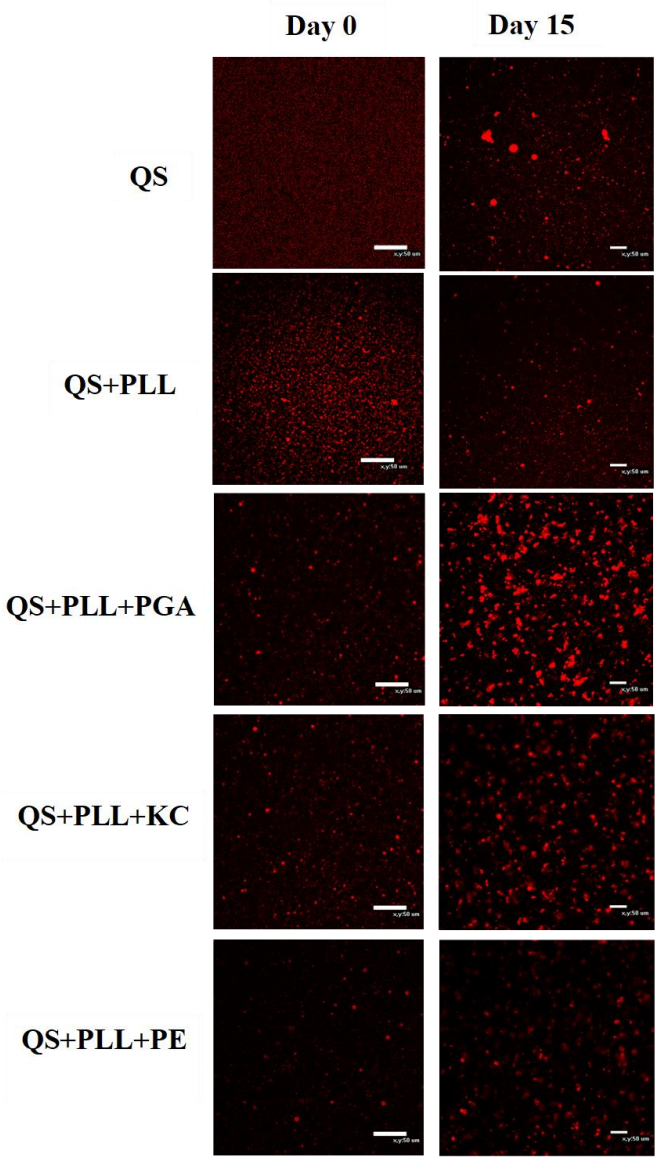


Figure 5.5 Confocal microscopy images impressions of β-carotene-loaded primary, secondary, and tertiary nanoemulsions before and after storage at 55°C for 15 days.

5.4. Conclusion

These studies showed that the resistance of β-carotene to degradation in multilayer nanoemulsions depending on the composition of the nanolaminated coating formed around

the droplets. In particular, when the outer layer was negatively charged, the rate of carotenoid degradation was appreciable faster than when it was positively charged. This effect was mainly attributed to the electrostatic repulsion of cationic transition metal ions away from the droplet surfaces by cationic biopolymers. However, there may be other problems associated with creating cationic oil droplets for application in some foods. For instance, cationic oil droplets may interact with other anionic ingredients in foods, thereby causing precipitation. Alternatively, cationic oil droplets may interact with anionic mucin molecules in the human mouth, which could lead to an undesirable mouthfeel, such as astringency. Consequently, further research is required to determine how these different nanoemulsions behave in real food products.

CHAPTER 6

6. CONCLUSION AND FUTURE STUDIES

This study has shown that multilayer nanoemulsions can be used to encapsulate lipophilic bioactive ingredients. The stability and physiochemical properties of these nanoemulsions depended on the nature of the emulsifiers and biopolymers used to create the multilayer coatings. Consequently, they must be carefully designed for specific food applications, depending on solution and environmental conditions. For instance, it was found that under highly acidic conditions (pH 2.0), nanoemulsions with an outer saponin layer need to be protected from flocculation by coating them with one or two biopolymer layers. It was also found that when the surrounding solution had a high ionic strength, the out PLL layer in secondary nanoemulsions tends to detach from the droplets because of weakening of the electrostatic attractive forces. This phenomenon could be used for creating triggered or controlled release systems for encapsulated agents in the human body. Lastly, multilayer nanoemulsions were shown to have good resistance to heating (90ºC, 30 min), which may be useful for applications where food or beverage products experience high temperatures.

Based on the current findings, a number of future studies can be proposed to further develop these multilayer nanoemulsions:

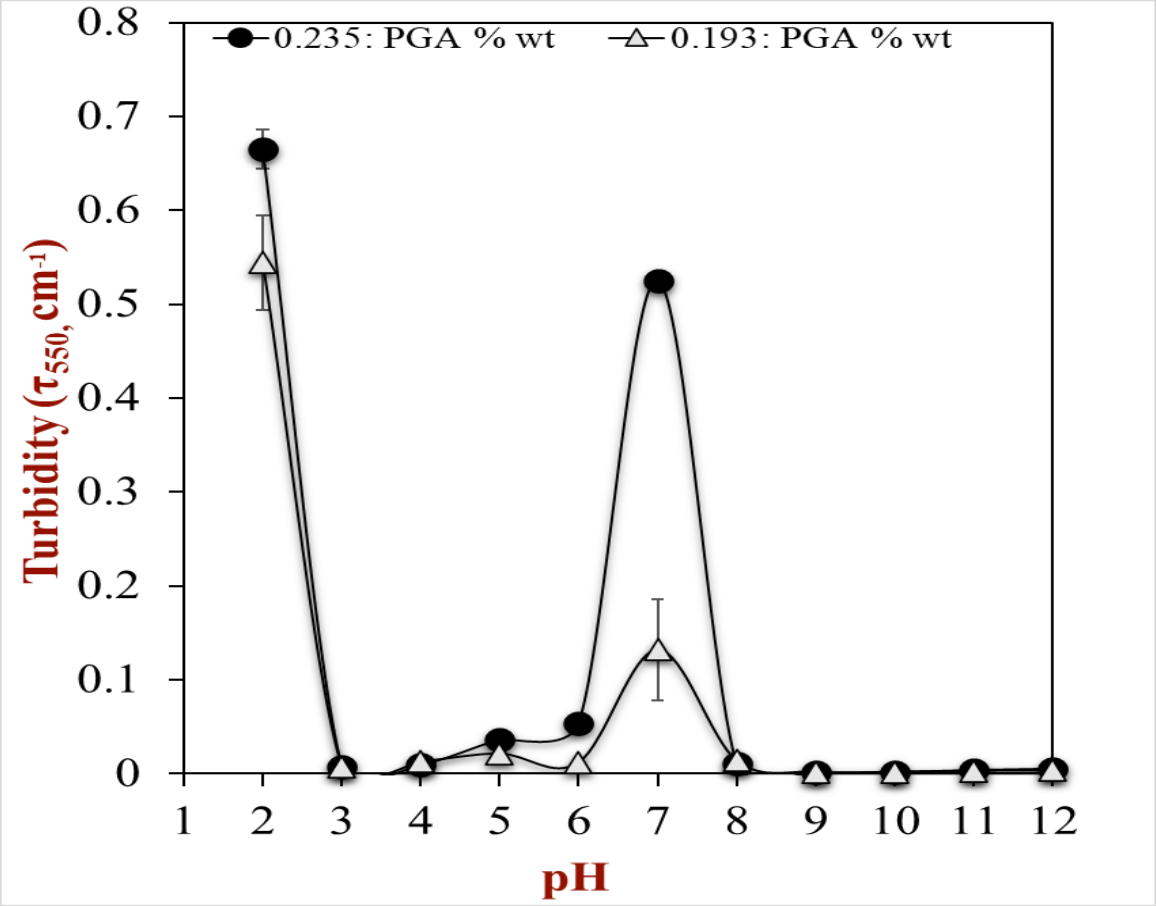
- The addition of natural antioxidants to improve the stability of encapsulated bioactive agents would be useful. In particular, identifying the location and mode of action of these antioxidants would be important.

- An improved understanding of the gastrointestinal fate of multilayer nanoemulsions would also be beneficial. This could be achieved by passing them through a simulated gastrointestinal tract or by using animal feeding studies. The impact of

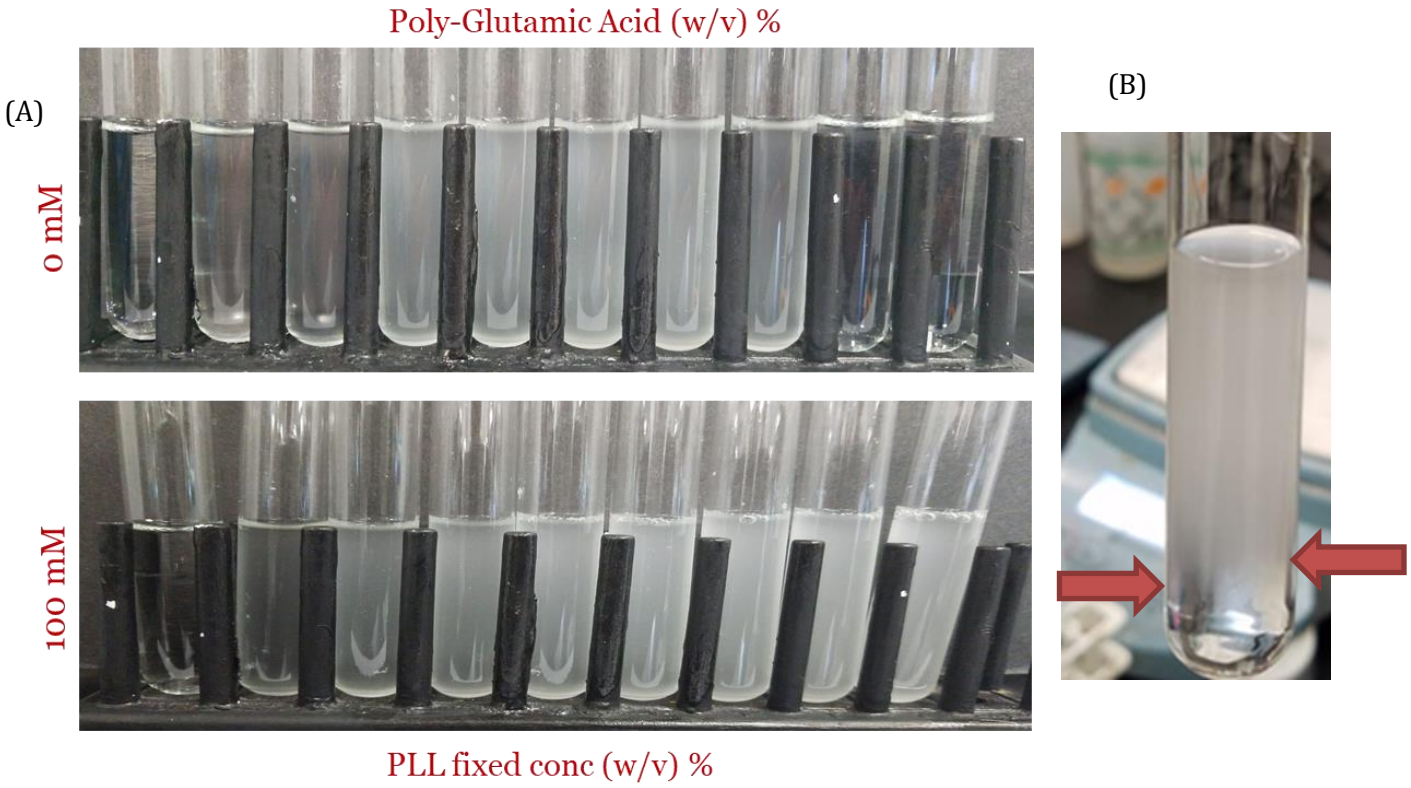
the multilayer coatings on the rate of lipid digestion and the release of encapsulated components would be useful.

- An improved understanding of the behavior of multilayer nanoemulsions in real food products would also be useful. For instance, it would be interesting to determine the multilayer coatings remain intact or become detached from the droplet surfaces, as well as the implications of this phenomenon on the stability and functional performance of the nanoemulsions.

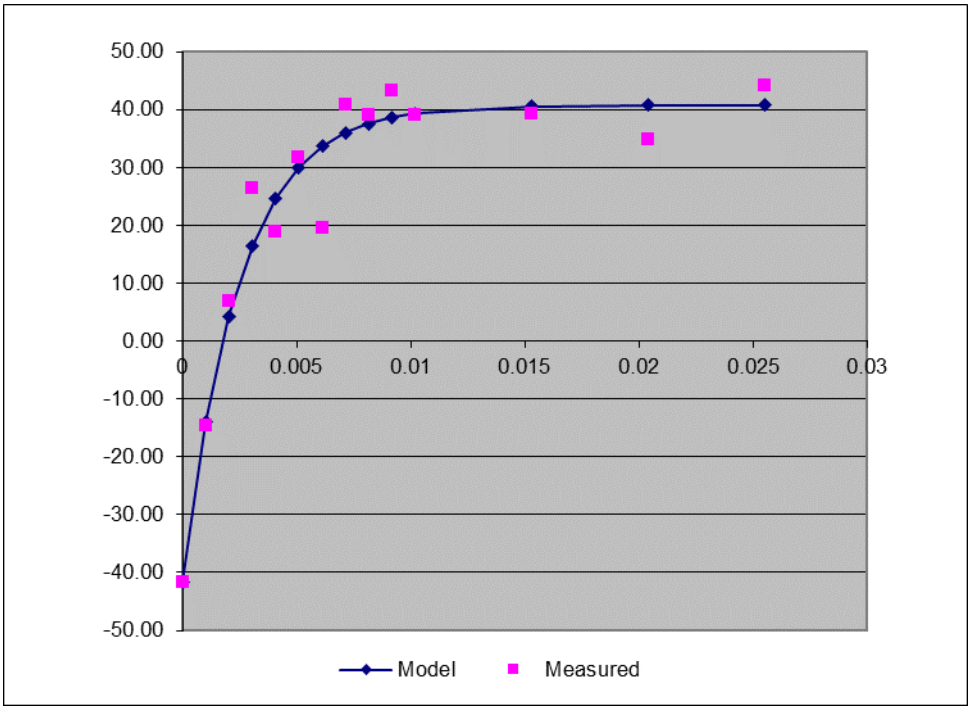
APPENDIX



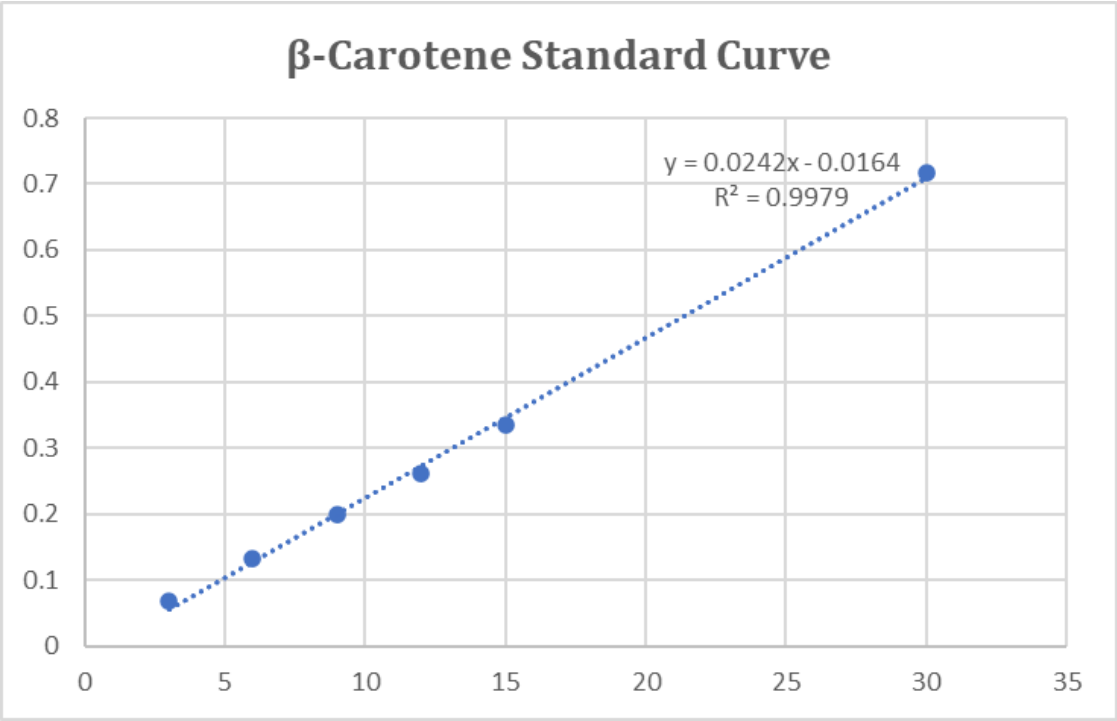
S1. Turbidity UV-Vis (550nm) measurements of two selected samples; (0.235%wt PGA) with (0.193%wt PGA) when they were exposed to different pH conditions.



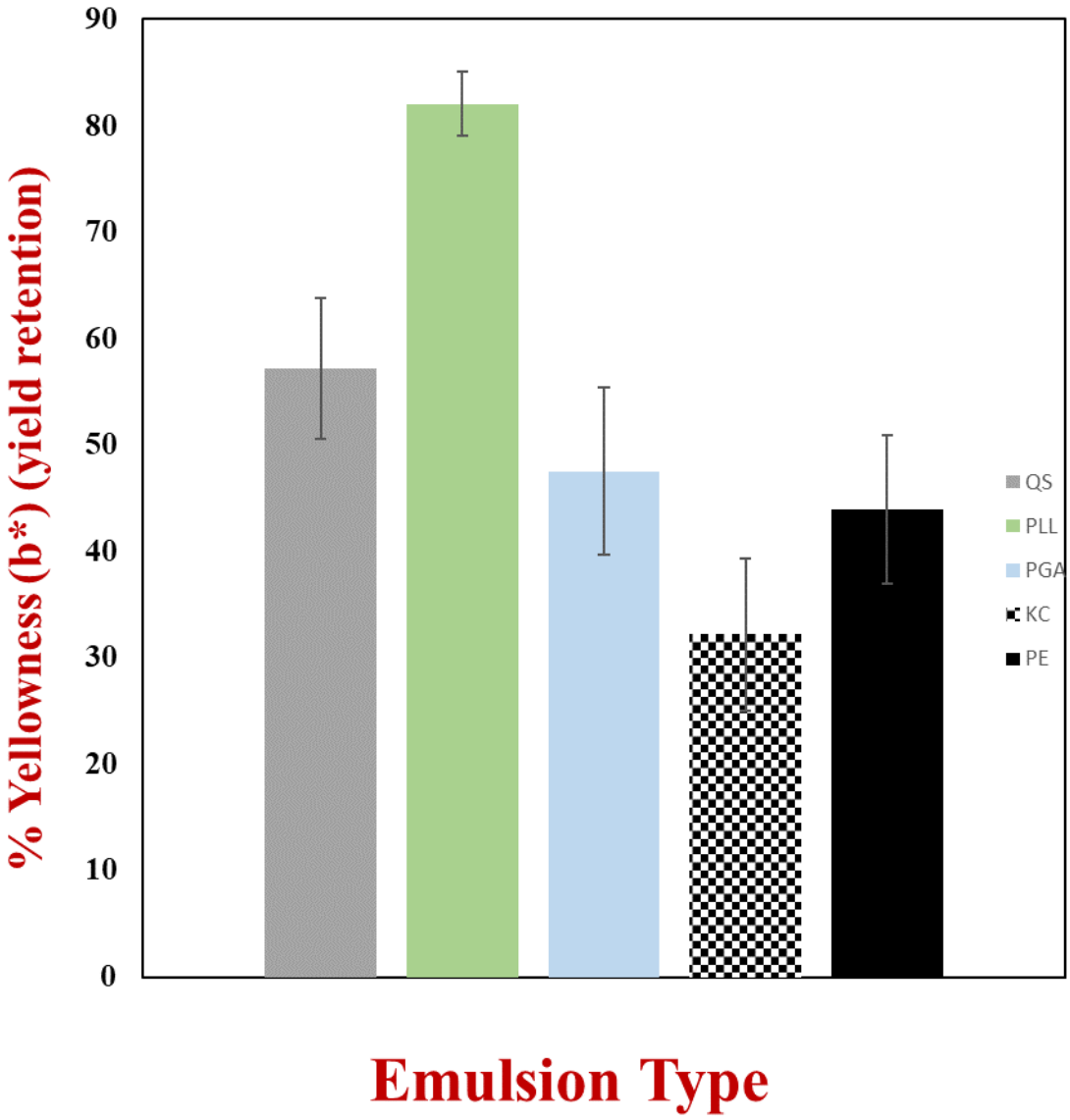
S2. Visual Appearances of: (A) Electrostatic repulsions screenings influence by different Ionic Strength (monovalent salts (NaCl)) concentrations. (B) After two attractive polyelectrolytes are mixed together, the dense liquid phase formed, which is relatively concentrated in both polyelectrolytes, is called the coacervate



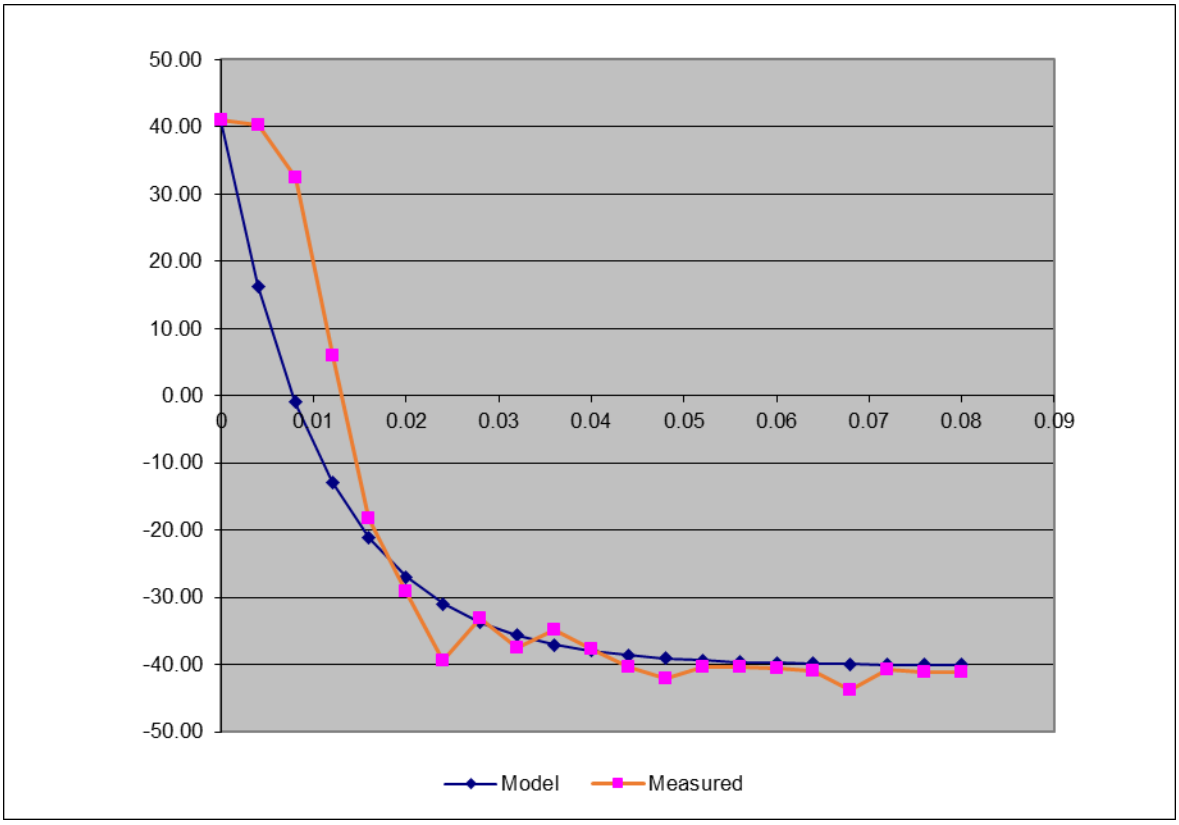
S3. Original "Model vs Measured" ζ-potential (mV) of cationic Poly-L-lysine (PLL) second layer, electrostatic deposition onto anionic Quillaja saponin (QS) nanoemulsions droplet surfaces-membranes.



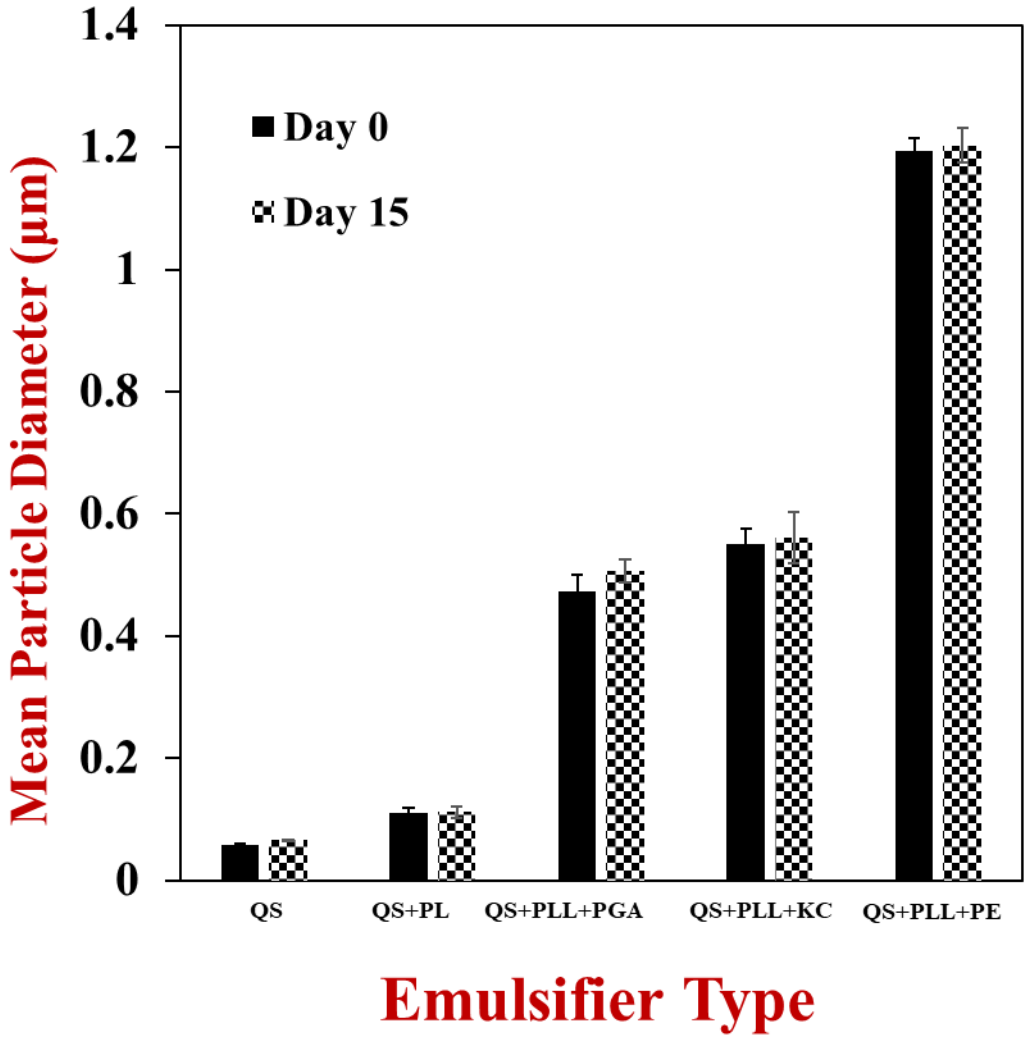
S4. β-Carotene (mcg/mL) standard curve, utilized for *chapter #5* experiments.



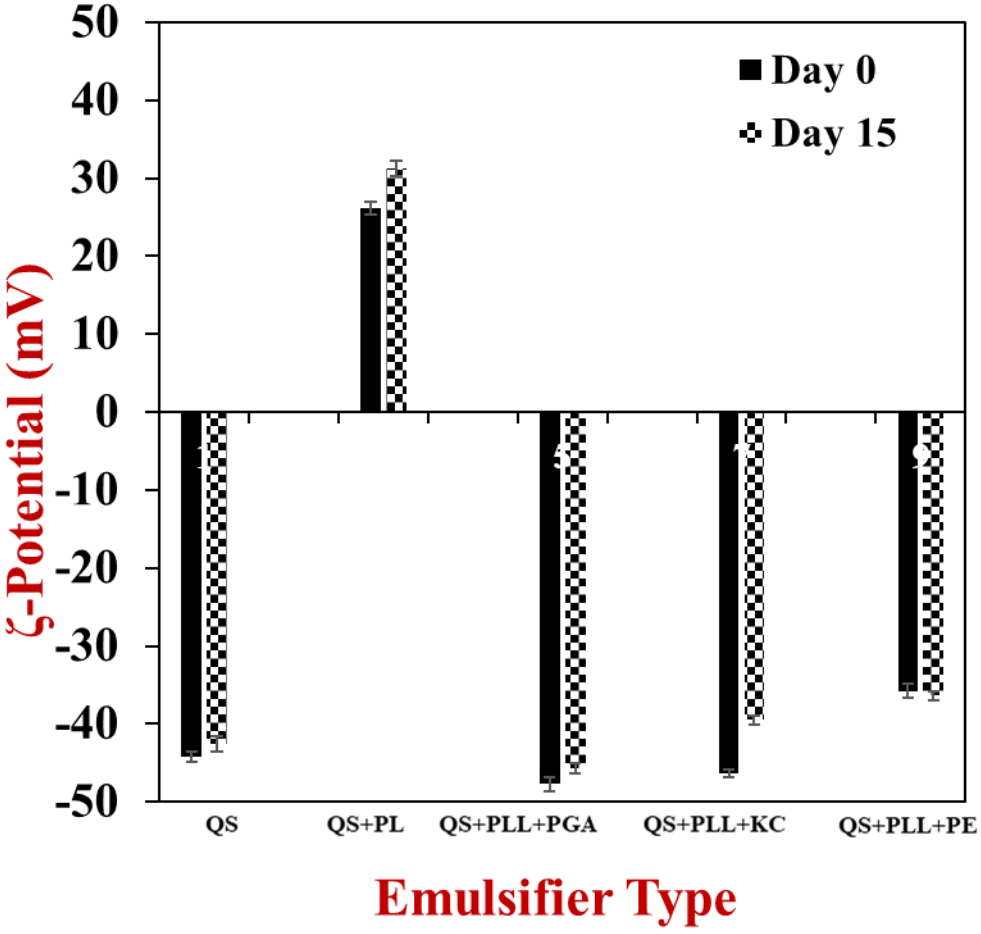
S5. β-Carotene % (Yellowness, b*) color retention yield from QS, PLL, PGA, KC and PE respective layers.



S6. Original "Model vs Measured" ζ-potential (mV) of anionic Pectin (PE) tertiary layer, electrostatic deposition onto secondary cationic Poly-L-lysine (PLL)-Quillaja saponin (QS) nanoemulsions droplet surfaces-membranes.



S7. Particle Size comparison between Day0 to Day15 of β-Carotene primary, secondary and tertiary nanoemulsions.



S8. Particle Surface Charges (ζ-potential) comparison between Day0 to Day15 of β-Carotene primary, secondary and tertiary nanoemulsions. Primary (QS) and tertiary layers (PGA, KC, PE) shown a strong anionic electrostatic behavior, while (PLL) secondary layer was the cationic counterpart layer.

BIBLIOGRAPHY.

1. Szyk-Warszyńska, L.; Piekoszewska, J.; Warszyński, P., Formation and stability of poly-L-lysine/casein multilayers. *Adsorption* **2010,** *16* (4-5), 241-248.

2. Szczepanowicz, K.; Hoel, H. J.; Szyk-Warszynska, L.; Bielańska, E.; Bouzga, A. M.; Gaudernack, G.; Simon, C.; Warszynski, P., Formation of Biocompatible Nanocapsules with Emulsion Core and Pegylated Shell by Polyelectrolyte Multilayer Adsorption. *Langmuir* **2010,** *26* (15), 12592-12597.

3. Guzey, D.; McClements, D. J., Formation, stability and properties of multilayer emulsions for application in the food industry. *Advances in colloid and interface science* **2006,** *128*, 227-248.

4. Decher, G.; Schlenoff, J., *Multilayer thin films*. Wiley Online Library: 2012.

5. Antunes, J. C.; Pereira, C. L.; Molinos, M.; Ferreira-da-Silva, F.; Dessì, M.; Gloria, A.; Ambrosio, L.; Gonçalves, R. M.; Barbosa, M. r. A., Layer-by-layer self-assembly of chitosan and poly (γ-glutamic acid) into polyelectrolyte complexes. *Biomacromolecules* **2011,** *12* (12), 4183-4195.

6. Steitz, R.; Leiner, V.; Siebrecht, R.; Klitzing, R. v., Influence of the ionic strength on the structure of polyelectrolyte films at the solid/liquid interface. *Colloids and Surfaces A: physicochemical and engineering aspects* **2000,** *163* (1), 63-70.

7. Diamanti, E.; Muzzio, N.; Gregurec, D.; Irigoyen, J.; Pasquale, M.; Azzaroni, O.; Brinkmann, M.; Moya, S. E., Impact of thermal annealing on wettability and antifouling characteristics of alginate poly-l-lysine polyelectrolyte multilayer films. *Colloids and Surfaces B: Biointerfaces* **2016,** *145*, 328-337.

8. Guzey, D.; McClements, D. J., Impact of electrostatic interactions on formation and stability of emulsions containing oil droplets coated by β-lactoglobulin− pectin complexes. *Journal of agricultural and food chemistry* **2007,** *55* (2), 475-485.

9. Reineccius, G., Use of proteins for the delivery of flavours and other bioactive compounds. *Food Hydrocolloids* **2019,** *86*, 62-69.

10. Iwashita, K.; Handa, A.; Shiraki, K., Coacervates and coaggregates: Liquid–liquid and liquid–solid phase transitions by native and unfolded protein complexes. *International journal of biological macromolecules* **2018,** *120*, 10-18.

11. Kizilay, E.; Kayitmazer, A. B.; Dubin, P. L., Complexation and coacervation of polyelectrolytes with oppositely charged colloids. *Advances in colloid and interface science* **2011,** *167* (1-2), 24-37.

12. Obermeyer, A. C.; Mills, C. E.; Dong, X.-H.; Flores, R. J.; Olsen, B. D., Complex coacervation of supercharged proteins with polyelectrolytes. *Soft Matter* **2016,** *12* (15), 3570-3581.

13. Güzey, D.; McClements, D. J., Influence of environmental stresses on O/W emulsions stabilized by β-lactoglobulin–pectin and β-lactoglobulin–pectin–chitosan membranes produced by the electrostatic layer-by-layer deposition technique. *Food Biophysics* **2006,** *1* (1), 30-40.

14. Dickinson, E., Food emulsions and foams: Stabilization by particles. *Current Opinion in Colloid & Interface Science* **2010,** *15* (1-2), 40-49.

15. McClements, D. J., Theoretical analysis of factors affecting the formation and stability of multilayered colloidal dispersions. *Langmuir* **2005,** *21* (21), 9777-9785.

16. McClements, D. J., Food emulsions: principles, practices, and techniques. CRC press: 2015.

17. Panchagnula, V.; Jeon, J.; Rusling, J. F.; Dobrynin, A. V., Molecular dynamics simulations of polyelectrolyte multilayering on a charged particle. *Langmuir* **2005,** *21* (3), 1118-1125.

18. Muriel Mundo, J. L.; Liu, J.; Tan, Y.; Zhou, H.; Zhang, Z.; Julian McClements, D., Characterization of electrostatic interactions and complex formation of ɣ-poly-glutamic acid (PGA) and ε-poly-l-lysine (PLL) in aqueous solutions. *Food Research International* **2019**, 108781.

19. Muriel Mundo, J. L.; Zhou, H.; Tan, Y.; Liu, J.; McClements, D. J., Stabilization of soybean oil-in-water emulsions using polypeptide multilayers: Cationic polylysine and anionic polyglutamic acid. *Food Research International* **2020,** *137*, 109304.

20. Mundo, J. L. M.; Zhou, H.; Tan, Y.; Liu, J.; McClements, D. J., Enhancing emulsion functionality using multilayer technology: Coating lipid droplets with saponin-polypeptide-polysaccharide layers by electrostatic deposition. *Food Research International* **2020**, 109864.

21. Ogawa, S.; Decker, E. A.; McClements, D. J., Influence of environmental conditions on the stability of oil in water emulsions containing droplets stabilized by lecithin− chitosan membranes. *Journal of agricultural and food chemistry* **2003,** *51* (18), 5522-5527.

22. Surh, J.; Decker, E. A.; McClements, D. J., Utilisation of spontaneous emulsification to fabricate lutein-loaded nanoemulsion-based delivery systems: factors influencing particle size and colour. *International Journal of Food Science & Technology* **2017,** *52* (6), 1408-1416.

23. Bortnowska, G., Multilayer oil-in-water emulsions: formation, characteristics and application as the carriers for lipophilic bioactive food components–a review. *Polish journal of food and nutrition sciences* **2015,** *65* (3), 157-166.

24. McClements, D. J., Nanoscale nutrient delivery systems for food applications: improving bioactive dispersibility, stability, and bioavailability. *Journal of food science* **2015,** *80* (7), N1602-N1611.

25. Mao, Y.; Dubot, M.; Xiao, H.; McClements, D. J., Interfacial engineering using mixed protein systems: Emulsion-based delivery systems for encapsulation and stabilization of β-carotene. *Journal of agricultural and food chemistry* **2013,** *61* (21), 5163-5169.

26. Li, Y.; Hu, M.; Xiao, H.; Du, Y.; Decker, E. A.; McClements, D. J., Controlling the functional performance of emulsion-based delivery systems using multi-component biopolymer coatings. *European Journal of Pharmaceutics and Biopharmaceutics* **2010,** *76* (1), 38-47.

27. Klinkesorn, U.; Sophanodora, P.; Chinachoti, P.; McClements, D. J.; Decker, E. A., Increasing the oxidative stability of liquid and dried tuna oil-in-water emulsions with electrostatic layer-by-layer deposition technology. *Journal of agricultural and food chemistry* **2005,** *53* (11), 4561-4566.

28. Klinkesorn, U.; McClements, D. J., Impact of lipase, bile salts, and polysaccharides on properties and digestibility of tuna oil multilayer emulsions stabilized by lecithin–chitosan. *Food Biophysics* **2010,** *5* (2), 73-81.

29. Iwanaga, D.; Gray, D.; Decker, E. A.; Weiss, J.; McClements, D. J., Stabilization of soybean oil bodies using protective pectin coatings formed by electrostatic deposition. *Journal of agricultural and food chemistry* **2008,** *56* (6), 2240-2245.

30. Crouzier, T.; Boudou, T.; Picart, C., Polysaccharide-based polyelectrolyte multilayers. *Current Opinion in Colloid & Interface Science* **2010,** *15* (6), 417-426.

31. Dragan, E. S.; Dinu, M. V., Polysaccharides constructed hydrogels as vehicles for proteins and peptides. A review. *Carbohydrate Polymers* **2019,** *225*, 115210.

32. Espinal-Ruiz, M.; Parada-Alfonso, F.; Restrepo-Sánchez, L.-P.; Narváez-Cuenca, C.-E.; McClements, D. J., Impact of dietary fibers [methyl cellulose, chitosan, and pectin] on digestion of lipids under simulated gastrointestinal conditions. *Food & Function* **2014,** *5* (12), 3083-3095.

33. Gunness, P.; Gidley, M. J., Mechanisms underlying the cholesterol-lowering properties of soluble dietary fibre polysaccharides. *Food & function* **2010,** *1* (2), 149-155.

34. Fabek, H.; Messerschmidt, S.; Brulport, V.; Goff, H. D., The effect of in vitro digestive processes on

the viscosity of dietary fibres and their influence on glucose diffusion. *Food Hydrocolloids* **2014,** *35*, 718-726.

35. Kristensen, M.; Jensen, M. G., Dietary fibres in the regulation of appetite and food intake. Importance of viscosity. *Appetite* **2011,** *56* (1), 65-70.

36. Li, Y.; McClements, D. J., Modulating lipid droplet intestinal lipolysis by electrostatic complexation with anionic polysaccharides: Influence of cosurfactants. *Food Hydrocolloids* **2014,** *35*, 367-374.

37. Simo, O. K.; Mao, Y.; Tokle, T.; Decker, E. A.; McClements, D. J., Novel strategies for fabricating reduced fat foods: Heteroaggregation of lipid droplets with polysaccharides. *Food research international* **2012,** *48* (2), 337-345.

38. Tokle, T.; Lesmes, U.; McClements, D. J., Impact of electrostatic deposition of anionic polysaccharides on the stability of oil droplets coated by lactoferrin. *Journal of agricultural and food chemistry* **2010,** *58* (17), 9825-9832.

39. Hur, S. J.; Lim, B. O.; Decker, E. A.; McClements, D. J., In vitro human digestion models for food applications. *Food chemistry* **2011,** *125* (1), 1-12.

40. McClements, D. J.; Li, Y., Review of in vitro digestion models for rapid screening of emulsion-based systems. *Food & function* **2010,** *1* (1), 32-59.

41. Beysseriat, M.; Decker, E. A.; McClements, D. J., Preliminary study of the influence of dietary fiber on the properties of oil-in-water emulsions passing through an in vitro human digestion model. *Food Hydrocolloids* **2006,** *20* (6), 800-809.

42. Brownlee, I. A., The physiological roles of dietary fibre. *Food hydrocolloids* **2011,** *25* (2), 238-250.

43. Castillo, M.; Martín-Orúe, S.; Anguita, M.; Pérez, J.; Gasa, J., Adaptation of gut microbiota to corn physical structure and different types of dietary fibre. *Livestock Science* **2007,** *109* (1-3), 149-152.

44. Anal, A. K.; Bhopatkar, D.; Tokura, S.; Tamura, H.; Stevens, W. F., Chitosan-alginate multilayer beads for gastric passage and controlled intestinal release of protein. *Drug development and industrial pharmacy* **2003,** *29* (6), 713-724.

45. Leroux, J.; Langendorff, V.; Schick, G.; Vaishnav, V.; Mazoyer, J., Emulsion stabilizing properties of pectin. *Food Hydrocolloids* **2003,** *17* (4), 455-462.

46. Lopez-Pena, C. L.; McClements, D. J., Optimizing delivery systems for cationic biopolymers: Competitive interactions of cationic polylysine with anionic κ-carrageenan and pectin. *Food chemistry* **2014,** *153*, 9-14.

47. Gu, Y. S.; Decker, E. A.; McClements, D. J., Influence of pH and carrageenan type on properties of β-lactoglobulin stabilized oil-in-water emulsions. *Food Hydrocolloids* **2005,** *19* (1), 83-91.

48. Dickinson, E.; Pawlowsky, K., Effect of ι-carrageenan on flocculation, creaming, and rheology of a protein-stabilized emulsion. *Journal of agricultural and food chemistry* **1997,** *45* (10), 3799-3806.

49. Anal, A. K.; Stevens, W. F., Chitosan–alginate multilayer beads for controlled release of ampicillin. *International journal of pharmaceutics* **2005,** *290* (1-2), 45-54.

50. Yuan, W.; Dong, H.; Li, C. M.; Cui, X.; Yu, L.; Lu, Z.; Zhou, Q., pH-Controlled Construction of Chitosan/Alginate Multilayer Film: Characterization and Application for Antibody Immobilization. *Langmuir* **2007,** *23* (26), 13046-13052.

51. Acevedo-Fani, A.; Silva, H. D.; Soliva-Fortuny, R.; Martín-Belloso, O.; Vicente, A. A., Formation, stability and antioxidant activity of food-grade multilayer emulsions containing resveratrol. *Food Hydrocolloids* **2017,** *71*, 207-215.

52. Yuan, W.; Dong, H.; Li, C. M.; Cui, X.; Yu, L.; Lu, Z.; Zhou, Q., pH-controlled construction of chitosan/alginate multilayer film: characterization and application for antibody immobilization. *Langmuir* **2007,** *23* (26), 13046-13052.

53. Butstraen, C.; Salaün, F., Preparation of microcapsules by complex coacervation of gum Arabic and chitosan. *Carbohydrate Polymers* **2014,** *99*, 608-616.

54. Fäldt, P.; Bergenståhl, B.; Claesson, P. M., Stabilization by chitosan of soybean oil emulsions coated with phospholipid and glycocholic acid. *Colloids and surfaces A: Physicochemical and engineering aspects* **1993,** *71* (2), 187-195.

55. Hu, M.; McClements, D. J.; Decker, E. A., Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. *Journal of agricultural and food chemistry* **2003,** *51* (5), 1435-1439.

56. Hu, M.; McClements, D. J.; Decker, E. A., Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. *Journal of agricultural and food chemistry* **2003,** *51* (6), 1696-1700.

57. Coupland, J. N.; McClements, D. J., Lipid oxidation in food emulsions. *Trends in Food Science & Technology* **1996,** *7* (3), 83-91.

58. Lacatusu, I.; Mitrea, E.; Badea, N.; Stan, R.; Oprea, O.; Meghea, A., Lipid nanoparticles based on omega-3 fatty acids as effective carriers for lutein delivery. Preparation and in vitro characterization studies. *Journal of Functional Foods* **2013,** *5* (3), 1260-1269.

59. Fustier, P.; Achouri, A.; Taherian, A. R.; Britten, M.; Pelletier, M. n.; Sabik, H.; Villeneuve, S. b.; Mondor, M., Protein–protein multilayer oil-in-water emulsions for the microencapsulation of flaxseed oil: Effect of whey and fish gelatin concentration. *Journal of agricultural and food chemistry* **2015,** *63* (42), 9239-9250.

60. Gudipati, V.; Sandra, S.; McClements, D. J.; Decker, E. A., Oxidative stability and in vitro digestibility of fish oil-in-water emulsions containing multilayered membranes. *Journal of agricultural and food chemistry* **2010,** *58* (13), 8093-8099.

61. Xu, D.; Aihemaiti, Z.; Cao, Y.; Teng, C.; Li, X., Physicochemical stability, microrheological properties and microstructure of lutein emulsions stabilized by multilayer membranes consisting of whey protein isolate, flaxseed gum and chitosan. *Food Chemistry* **2016,** *202*, 156-164.

62. Santos, O.; Nylander, T.; Paulsson, M.; Trägårdh, C., Whey protein adsorption onto steel surfaces—effect of temperature, flow rate, residence time and aggregation. *Journal of Food Engineering* **2006,** *74* (4), 468-483.

63. Szyk-Warszyńska, L.; Kilan, K.; Socha, R. P., Characterization of casein and poly-l-arginine multilayer films. *Journal of Colloid and Interface Science* **2014,** *423*, 76-84.

64. Chen, F.; Liang, L.; Zhang, Z.; Deng, Z.; Decker, E. A.; McClements, D. J., Inhibition of lipid oxidation in nanoemulsions and filled microgels fortified with omega-3 fatty acids using casein as a natural antioxidant. *Food Hydrocolloids* **2017,** *63*, 240-248.

65. Maheshwari, R. K.; Singh, A. K.; Gaddipati, J.; Srimal, R. C., Multiple biological activities of curcumin: a short review. *Life sciences* **2006,** *78* (18), 2081-2087.

66. Szyk-Warszyńska, L.; Gergely, C.; Jarek, E.; Cuisinier, F.; Socha, R. P.; Warszyński, P., Calcium uptake by casein embedded in polyelectrolyte multilayer. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2009,** *343* (1), 118-126.

67. García-Montoya, I. A.; Cendón, T. S.; Arévalo-Gallegos, S.; Rascón-Cruz, Q., Lactoferrin a multiple bioactive protein: an overview. *Biochimica et Biophysica Acta (BBA)-General Subjects* **2012,** *1820* (3), 226-236.

68. Ye, A.; Singh, H., Adsorption behaviour of lactoferrin in oil-in-water emulsions as influenced by interactions with β-lactoglobulin. *Journal of Colloid and Interface Science* **2006,** *295* (1), 249-254.

69. Boland, M.; Singh, H., *Milk proteins: from expression to food*. Academic Press: 2019.

70. Liu, F.; Ma, C.; Zhang, R.; Gao, Y.; McClements, D. J., Controlling the potential gastrointestinal fate of β-carotene emulsions using interfacial engineering: Impact of coating lipid droplets with polyphenol-protein-carbohydrate conjugate. *Food chemistry* **2017,** *221*, 395-403.

71. Liu, F.; Wang, D.; Sun, C.; McClements, D. J.; Gao, Y., Utilization of interfacial engineering to improve physicochemical stability of β-carotene emulsions: Multilayer coatings formed using protein and protein–polyphenol conjugates. *Food Chemistry* **2016,** *205*, 129-139.

72. Jones, O.; Decker, E. A.; McClements, D. J., Thermal analysis of β-lactoglobulin complexes with pectins or carrageenan for production of stable biopolymer particles. *Food Hydrocolloids* **2010,** *24* (2-3), 239-248.

73. Adams, J. J.; Anderson, B. F.; Norris, G. E.; Creamer, L. K.; Jameson, G. B., Structure of bovine β-lactoglobulin (variant A) at very low ionic strength. *Journal of structural biology* **2006,** *154* (3), 246-254.

74. Hoffmann, M. A.; van Mil, P. J., Heat-induced aggregation of β-lactoglobulin as a function of pH. *Journal of agricultural and food chemistry* **1999,** *47* (5), 1898-1905.

75. Moreau, L.; Kim, H.-J.; Decker, E. A.; McClements, D. J., Production and characterization of oil-in-water emulsions containing droplets stabilized by β-lactoglobulin− pectin membranes. *Journal of agricultural and food chemistry* **2003,** *51* (22), 6612-6617.

76. Guzey, D.; Kim, H.; McClements, D., Factors influencing the production of o/w emulsions stabilized by β-lactoglobulin–pectin membranes. *Food Hydrocolloids* **2004,** *18* (6), 967-975.

77. McClements, D. J., Nanoparticle-and microparticle-based delivery systems: Encapsulation, protection and release of active compounds. CRC press: 2014.

78. Burgos-Díaz, C.; Wandersleben, T.; Marqués, A. M.; Rubilar, M., Multilayer emulsions stabilized by vegetable proteins and polysaccharides. *Current Opinion in Colloid & Interface Science* **2016,** *25*, 51-57.

79. Zhou, J.; Wang, B.; Tong, W.; Maltseva, E.; Zhang, G.; Krastev, R.; Gao, C.; Möhwald, H.; Shen, J., Influence of assembling pH on the stability of poly (l-glutamic acid) and poly (l-lysine) multilayers against urea treatment. *Colloids and Surfaces B: Biointerfaces* **2008,** *62* (2), 250-257.

80. Carpenter, J.; George, S.; Saharan, V. K., Curcumin encapsulation in multilayer oil-in-water emulsion: synthesis using ultrasonication and studies on stability and antioxidant and release activities. *Langmuir* **2019,** *35* (33), 10866-10876.

81. Hamidon, N.; Zaidel, D.; El-Enshasy, H., Stabilization of Multilayer Emulsion as Influenced by Sweet Potato Pectin Concentration, pH and Salt. **2019**.

82. Aoki, T.; Decker, E. A.; McClements, D. J., Influence of environmental stresses on stability of O/W emulsions containing droplets stabilized by multilayered membranes produced by a layer-by-layer electrostatic deposition technique. *Food Hydrocolloids* **2005,** *19* (2), 209-220.

83. Burke, S. E.; Barrett, C. J., Acid− base equilibria of weak polyelectrolytes in multilayer thin films. *Langmuir* **2003,** *19* (8), 3297-3303.

84. McClements, D. J.; Decker, E. A., Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of food science* **2000,** *65* (8), 1270-1282.

85. Ushikubo, F. Y.; Cunha, R. L., Stability mechanisms of liquid water-in-oil emulsions. *Food Hydrocolloids* **2014,** *34*, 145-153.

86. Kato, N.; Schuetz, P.; Fery, A.; Caruso, F., Thin multilayer films of weak polyelectrolytes on colloid particles. *Macromolecules* **2002,** *35* (26), 9780-9787.

87. Waraho, T.; Cardenia, V.; Rodriguez-Estrada, M. T.; McClements, D. J.; Decker, E. A., Prooxidant Mechanisms of Free Fatty Acids in Stripped Soybean Oil-in-Water Emulsions. *Journal of agricultural and food chemistry* **2009,** *57* (15), 7112-7117.

88. Benjamin, O.; Silcock, P.; Leus, M.; Everett, D., Multilayer emulsions as delivery systems for controlled release of volatile compounds using pH and salt triggers. *Food Hydrocolloids* **2012,** *27* (1), 109-118.

89. Domard, A.; Rinaudo, M., Polyelectrolyte complexes. Interaction of poly (L-lysine)-poly (L-glutamic acid) in dilute aqueous solution. *Macromolecules* **1980,** *13* (4), 898-904.

90. Van der Gucht, J.; Spruijt, E.; Lemmers, M.; Stuart, M. A. C., Polyelectrolyte complexes: Bulk phases and colloidal systems. *Journal of colloid and interface science* **2011,** *361* (2), 407-422.

91. Tan, C.; Selig, M. J.; Lee, M. C.; Abbaspourrad, A., Polyelectrolyte microcapsules built on CaCO3 scaffolds for the integration, encapsulation, and controlled release of copigmented anthocyanins. *Food Chemistry* **2018,** *246*, 305-312.

92. Ball, V.; Bernsmann, F.; Betscha, C.; Maechling, C.; Kauffmann, S.; Senger, B.; Voegel, J.-C.; Schaaf, P.; Benkirane-Jessel, N., Polyelectrolyte Multilayer Films Built from Poly(l-lysine) and a Two-Component Anionic Polysaccharide Blend. *Langmuir* **2009,** *25* (6), 3593-3600.

93. Barbosa-Cánovas, G. V.; Ortega-Rivas, E.; Juliano, P.; Yan, H., *Food powders: physical properties, processing, and functionality*. Springer: 2005; Vol. 86.

94. Shi, X.; Caruso, F., Release behavior of thin-walled microcapsules composed of polyelectrolyte multilayers. *Langmuir* **2001,** *17* (6), 2036-2042.

95. Chung, C.; Rojanasasithara, T.; Mutilangi, W.; McClements, D. J., Stability improvement of natural food colors: Impact of amino acid and peptide addition on anthocyanin stability in model beverages. *Food chemistry* **2017,** *218*, 277-284.

96. Brenes, C. H.; Del Pozo-Insfran, D.; Talcott, S. T., Stability of copigmented anthocyanins and ascorbic acid in a grape juice model system. *Journal of agricultural and food chemistry* **2005,** *53* (1), 49-56.

97. Han, J.; Zhang, Z.; Shang, W.; Yan, J.; Julian McClements, D.; Xiao, H.; Wu, H.; Zhu, B., Modulation of physicochemical stability and bioaccessibility of β-carotene using alginate beads and emulsion stabilized by scallop (Patinopecten yessoensis) gonad protein isolates. *Food Research International* **2020,** *129*, 108875.

98. Qian, C.; Decker, E. A.; Xiao, H.; McClements, D. J., Nanoemulsion delivery systems: Influence of carrier oil on β-carotene bioaccessibility. *Food Chemistry* **2012,** *135* (3), 1440-1447.

99. Salvia-Trujillo, L.; Qian, C.; Martín-Belloso, O.; McClements, D., Modulating β-carotene bioaccessibility by controlling oil composition and concentration in edible nanoemulsions. *Food chemistry* **2013,** *139* (1-4), 878-884.

100. Zhang, R.; Zhang, Z.; Zhang, H.; Decker, E. A.; McClements, D. J., Influence of emulsifier type on gastrointestinal fate of oil-in-water emulsions containing anionic dietary fiber (pectin). *Food Hydrocolloids* **2015,** *45*, 175-185.

101. Zhang, Z.; Zhang, R.; McClements, D. J., Encapsulation of β-carotene in alginate-based hydrogel beads: Impact on physicochemical stability and bioaccessibility. *Food Hydrocolloids* **2016,** *61*, 1-10.

102. Eghbal, N.; Choudhary, R., Complex coacervation: Encapsulation and controlled release of active agents in food systems. *LWT* **2018,** *90*, 254-264.

103. Kim, S.; Huang, J.; Lee, Y.; Dutta, S.; Yoo, H. Y.; Jung, Y. M.; Jho, Y.; Zeng, H.; Hwang, D. S., Complexation and coacervation of like-charged polyelectrolytes inspired by mussels. *Proceedings of the National Academy of Sciences* **2016,** *113* (7), E847-E853.

104. Nakajima, A.; Shinoda, K.; Hayashi, T.; Sato, H., Interactions between oppositely charged polypeptides. *Polymer Journal* **1975,** *7* (5), 550.

105. Perry, S.; Li, Y.; Priftis, D.; Leon, L.; Tirrell, M., The effect of salt on the complex coacervation of vinyl polyelectrolytes. *Polymers* **2014,** *6* (6), 1756-1772.

106. Priftis, D.; Leon, L.; Song, Z.; Perry, S. L.; Margossian, K. O.; Tropnikova, A.; Cheng, J.; Tirrell, M., Self-assembly of α-helical polypeptides driven by complex coacervation. *Angewandte Chemie International Edition* **2015,** *54* (38), 11128-11132.

107. Priftis, D.; Tirrell, M., Phase behaviour and complex coacervation of aqueous polypeptide solutions. *Soft Matter* **2012,** *8* (36), 9396-9405.

108. Spruijt, E.; Sprakel, J.; Stuart, M. A. C.; van der Gucht, J., Interfacial tension between a complex coacervate phase and its coexisting aqueous phase. *Soft Matter* **2010,** *6* (1), 172-178.

109. Weinbreck, F.; Minor, M.; De Kruif, C., Microencapsulation of oils using whey protein/gum arabic coacervates. *Journal of microencapsulation* **2004,** *21* (6), 667-679.

110. Dong, Z.; Ma, Y.; Hayat, K.; Jia, C.; Xia, S.; Zhang, X., Morphology and release profile of microcapsules encapsulating peppermint oil by complex coacervation. *Journal of Food Engineering* **2011,** *104* (3), 455-460.

111. Junyaprasert, V. B.; Mitrevej, A.; Sinchaipanid, N.; Boonme, P.; Wurster, D. E., Effect of process variables on the microencapsulation of vitamin A palmitate by gelatin-acacia coacervation. *Drug Development and Industrial Pharmacy* **2001,** *27* (6), 561-566.

112. Bajaj, I.; Singhal, R., Poly (glutamic acid)--an emerging biopolymer of commercial interest. *Bioresour Technol* **2011,** *102* (10), 5551-61.

113. Park, C.; Choi, J.-C.; Choi, Y.-H.; Nakamura, H.; Shimanouchi, K.; Horiuchi, T.; Misono, H.; Sewaki, T.; Soda, K.; Ashiuchi, M., Synthesis of super-high-molecular-weight poly-γ-glutamic acid by Bacillus subtilis subsp. chungkookjang. *Journal of Molecular Catalysis B: Enzymatic* **2005,** *35* (4-6), 128-133.

114. Wang, L.-L.; Chen, J.-T.; Wang, L.-F.; Wu, S.; Zhang, G.-z.; Yu, H.-Q.; Ye, X.-d.; Shi, Q.-S., Conformations and molecular interactions of poly-γ-glutamic acid as a soluble microbial product in aqueous solutions. *Scientific reports* **2017,** *7* (1), 12787.

115. Jamshidian, M.; Tehrany, E. A.; Imran, M.; Jacquot, M.; Desobry, S., Poly-lactic acid: production, applications, nanocomposites, and release studies. *Comprehensive reviews in food science and food safety* **2010,** *9* (5), 552-571.

116. Volodkin, D.; Ball, V.; Schaaf, P.; Voegel, J.-C.; Mohwald, H., Complexation of phosphocholine liposomes with polylysine. Stabilization by surface coverage versus aggregation. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **2007,** *1768* (2), 280-290.

117. Bankar, S. B.; Singhal, R. S., Panorama of poly-ε-lysine. **2013,** *3* (23), 8586-8603.

118. Chang, Y.; McLandsborough, L.; McClements, D. J., Interactions of a cationic antimicrobial (ε-polylysine) with an anionic biopolymer (pectin): an isothermal titration calorimetry, microelectrophoresis, and turbidity study. *Journal of agricultural and food chemistry* **2011,** *59* (10), 5579-5588.

119. Chang, Y.; McLandsborough, L.; McClements, D. J., Cationic antimicrobial (ε-polylysine)–anionic polysaccharide (pectin) interactions: influence of polymer charge on physical stability and antimicrobial efficacy. *Journal of agricultural and food chemistry* **2012,** *60* (7), 1837-1844.

120. Chang, Y.; McLandsborough, L.; McClements, D. J., Interaction of cationic antimicrobial (ε-polylysine) with food-grade biopolymers: Dextran, chitosan, carrageenan, alginate, and pectin. *Food research international* **2014,** *64*, 396-401.

121. Lopez-Pena, C. L.; McClements, D. J., Impact of a food-grade cationic biopolymer (ε-polylysine) on the digestion of emulsified lipids: In vitro study. *Food Research International* **2015,** *75*, 34-40.

122. Lopez-Pena, C. L.; Zheng, B.; Sela, D. A.; Decker, E. A.; Xiao, H.; McClements, D. J., Impact of ε-polylysine and pectin on the potential gastrointestinal fate of emulsified lipids: In vitro mouth, stomach and small intestine model. *Food chemistry* **2016,** *192*, 857-864.

123. Pandey, A. K.; Kumar, A., Improved microbial biosynthesis strategies and multifarious applications

of the natural biopolymer epsilon-poly-l-lysine. *Process Biochemistry* **2014,** *49* (3), 496-505.

124. Shih, I.-L.; Van, Y.-T.; Shen, M.-H., Biomedical applications of chemically and microbiologically synthesized poly (glutamic acid) and poly (lysine). *Mini reviews in medicinal chemistry* **2004,** *4* (2), 179-188.

125. Shih, L.; Shen, M.-H.; Van, Y.-T., Microbial synthesis of poly (ε-lysine) and its various applications. *Bioresource technology* **2006,** *97* (9), 1148-1159.

126. Wei, M.; Ge, Y.; Li, C.; Chen, Y.; Wang, W.; Duan, B.; Li, X., Antifungal activity of ε-poly-L-lysine on Trichothecium roseum in vitro and its mechanisms. *Physiological and Molecular Plant Pathology* **2018,** *103*, 23-27.

127. Dompé, M.; Cedano-Serrano, F. J.; Heckert, O.; van den Heuvel, N.; van der Gucht, J.; Tran, Y.; Hourdet, D.; Creton, C.; Kamperman, M., Thermoresponsive Complex Coacervate-Based Underwater Adhesive. *Advanced Materials* **2019**.

128. Shima, S.; Sakai, H., Polylysine produced by Streptomyces. *Agricultural and Biological Chemistry* **1977,** *41* (9), 1807-1809.

129. Shirling, E. T.; Gottlieb, D., Methods for characterization of Streptomyces species. *International journal of systematic bacteriology* **1966,** *16* (3), 313-340.

130. Bajaj, I.; Singhal, R., Poly (glutamic acid) – An emerging biopolymer of commercial interest. *Bioresource Technology* **2011,** *102* (10), 5551-5561.

131. Mundo, J. L. M.; Liu, J.; Tan, Y.; Zhou, H.; Zhang, Z.; McClements, D. J., Characterization of electrostatic interactions and complex formation of ɣ-poly-glutamic acid (PGA) and ε-poly-l-lysine (PLL) in aqueous solutions. *Food Research International* **2020,** *128*, 108781.

132. Hyldgaard, M.; Mygind, T.; Vad, B. S.; Stenvang, M.; Otzen, D. E.; Meyer, R. L., The Antimicrobial Mechanism of Action of Epsilon-Poly-l-Lysine. *Applied and Environmental Microbiology* **2014,** *80* (24), 7758.

133. Freire, E.; Mayorga, O. L.; Straume, M., Isothermal titration calorimetry. *Analytical chemistry* **1990,** *62* (18), 950A-959A.

134. Freyer, M. W.; Lewis, E. A., Isothermal Titration Calorimetry: Experimental Design, Data Analysis, and Probing Macromolecule/Ligand Binding and Kinetic Interactions. In *Methods in Cell Biology*, Academic Press: 2008; Vol. 84, pp 79-113.

135. Bhattacharyya, A.; Argillier, J., Microencapsulation by complex coacervation: effect of cationic surfactants. *Journal of Surface Science and Technology* **2005,** *21* (3/4), 161.

136. Tork, S. E.; Aly, M. M.; Alakilli, S. Y.; Al-Seeni, M. N., Purification and characterization of gamma poly glutamic acid from newly Bacillus licheniformis NRC20. *International Journal of Biological Macromolecules* **2015,** *74*, 382-391.

137. Chuah, A. M.; Kuroiwa, T.; Kobayashi, I.; Nakajima, M., Effect of chitosan on the stability and properties of modified lecithin stabilized oil-in-water monodisperse emulsion prepared by microchannel emulsification. *Food Hydrocolloids* **2009,** *23* (3), 600-610.

138. Fredrick, E.; Walstra, P.; Dewettinck, K., Factors governing partial coalescence in oil-in-water emulsions. *Advances in colloid and interface science* **2010,** *153* (1-2), 30-42.

139. Wang, B.; Wang, L.-j.; Li, D.; Adhikari, B.; Shi, J., Effect of gum Arabic on stability of oil-in-water emulsion stabilized by flaxseed and soybean protein. *Carbohydrate polymers* **2011,** *86* (1), 343-351.

140. Djordjevic, D.; Cercaci, L.; Alamed, J.; McClements, D. J.; Decker, E. A., Chemical and physical stability of citral and limonene in sodium dodecyl sulfate− chitosan and gum arabic-stabilized oil-in-water emulsions. *Journal of agricultural and food chemistry* **2007,** *55* (9), 3585-3591.

141. Lesmes, U.; Sandra, S.; Decker, E. A.; McClements, D. J., Impact of surface deposition of

lactoferrin on physical and chemical stability of omega-3 rich lipid droplets stabilised by caseinate. *Food Chemistry* **2010,** *123* (1), 99-106.

142. Hou, Z.; Gao, Y.; Yuan, F.; Liu, Y.; Li, C.; Xu, D., Investigation into the physicochemical stability and rheological properties of β-carotene emulsion stabilized by soybean soluble polysaccharides and chitosan. *Journal of agricultural and food chemistry* **2010,** *58* (15), 8604-8611.

143. Volodkin, D.; Ball, V.; Schaaf, P.; Voegel, J. C.; Mohwald, H., Complexation of phosphocholine liposomes with polylysine. Stabilization by surface coverage versus aggregation. *Biochimica Et Biophysica Acta-Biomembranes* **2007,** *1768* (2), 280-290.

144. Torchilin, V. P., Micellar nanocarriers: pharmaceutical perspectives. *Pharmaceutical research* **2007,** *24* (1), 1.

145. Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S. W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K., Liposome: classification, preparation, and applications. *Nanoscale research letters* **2013,** *8* (1), 102.

146. McClements, D. J., Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft matter* **2012,** *8* (6), 1719-1729.

147. Mishra, V.; Bansal, K. K.; Verma, A.; Yadav, N.; Thakur, S.; Sudhakar, K.; Rosenholm, J. M., Solid lipid nanoparticles: Emerging colloidal nano drug delivery systems. *Pharmaceutics* **2018,** *10* (4), 191.

148. McClements, D. J., Recent progress in hydrogel delivery systems for improving nutraceutical bioavailability. *Food Hydrocolloids* **2016**.

149. Ai, H.; Jones, S. A.; Lvov, Y. M., Biomedical applications of electrostatic layer-by-layer nano-assembly of polymers, enzymes, and nanoparticles. *Cell biochemistry and biophysics* **2003,** *39* (1), 23.

150. Schönhoff, M., Self-assembled polyelectrolyte multilayers. *Current opinion in colloid & interface science* **2003,** *8* (1), 86-95.

151. Poptoshev, E.; Schoeler, B.; Caruso, F., Influence of solvent quality on the growth of polyelectrolyte multilayers. *Langmuir* **2004,** *20* (3), 829-834.

152. Israelachvili, J. N., *Intermolecular and surface forces*. Academic press: 2011.

153. Schönhoff, M., Layered polyelectrolyte complexes: physics of formation and molecular properties. *Journal of Physics: Condensed Matter* **2003,** *15* (49), R1781.

154. Yang, Y.; Leser, M. E.; Sher, A. A.; McClements, D. J., Formation and stability of emulsions using a natural small molecule surfactant: Quillaja saponin (Q-Naturale®). *Food Hydrocolloids* **2013,** *30* (2), 589-596.

155. Comert, F.; Xu, A. Y.; Madro, S. P.; Liadinskaia, V.; Dubin, P. L., The so-called critical condition for polyelectrolyte-colloid complex formation. *The Journal of chemical physics* **2018,** *149* (16), 163321.

156. Evans, M.; Ratcliffe, I.; Williams, P. A., Emulsion stabilisation using polysaccharide-protein complexes. *Current Opinion in Colloid & Interface Science* **2013,** *18* (4), 272-282.

157. Grigoriev, D. O.; Miller, R., Mono- and multilayer covered drops as carriers. *Current Opinion in Colloid & Interface Science* **2009,** *14* (1), 48-59.

158. Shaw, L. A.; McClements, D. J.; Decker, E. A., Spray-dried multilayered emulsions as a delivery method for omega-3 fatty acids into food systems. *Journal of Agricultural and Food Chemistry* **2007,** *55* (8), 3112-3119.

159. Hamidon, N. H.; Zaidel, D. N. A.; El-Enshasy, H. A., Stabilization of Multilayer Emulsion as Influenced by Sweet Potato Pectin Concentration, pH and Salt. *Journal of Scientific & Industrial Research* **2019,** *78* (11), 775-781.

160. Zang, X. D.; Wang, J. W.; Yu, G. P.; Cheng, J. J., Addition of anionic polysaccharides to improve the stability of rice bran protein hydrolysate-stabilized emulsions. *Lwt-Food Science and Technology* **2019,** *111*, 573-581.

161. Mundo, J. L. M.; Zhou, H.; Tan, Y.; Liu, J.; McClements, D. J., Stabilization of soybean oil-in-water emulsions using polypeptide multilayers: Cationic polylysine and anionic polyglutamic acid. *Food Research International* **2020,** *Submitted*.

162. Maroziene, A.; De Kruif, C., Interaction of pectin and casein micelles. *Food hydrocolloids* **2000,** *14* (4), 391-394.

163. Sun, X.; Pan, C.; Ying, Z.; Yu, D.; Duan, X.; Huang, F.; Ling, J.; Ouyang, X.-k., Stabilization of zein nanoparticles with k-carrageenan and tween 80 for encapsulation of curcumin. *International Journal of Biological Macromolecules* **2020,** *146*, 549-559.

164. Kharat, M., Design and Fabrication of Colloidal Delivery Systems to Encapsulate and Protect Curcumin: An Important Hydrophobic Nutraceutical. **2020**.

165. Weinbreck, F.; Nieuwenhuijse, H.; Robijn, G. W.; De Kruif, C. G., Complexation of whey proteins with carrageenan. *Journal of agricultural and food chemistry* **2004,** *52* (11), 3550-3555.

166. Rochas, C.; Rinaudo, M., Mechanism of gel formation in κ-carrageenan. *Biopolymers: Original Research on Biomolecules* **1984,** *23* (4), 735-745.

167. Chronakis, I. S.; Doublier, J. L.; Piculell, L., Viscoelastic properties for kappa- and iota-carrageenan in aqueous NaI from the liquid-like to the solid-like behaviour. *Int J Biol Macromol* **2000,** *28* (1), 1-14.

168. de Aguiar, A. C.; de Paula, J. T.; Mundo, J. L. M.; Martínez, J.; McClements, D. J., Influence of type of natural emulsifier and microfluidization conditions on Capsicum oleoresin nanoemulsions properties and stability. *Journal of Food Process Engineering*, e13660.

169. Von Goeler, F.; Muthukumar, M., Adsorption of polyelectrolytes onto curved surfaces. *The Journal of chemical physics* **1994,** *100* (10), 7796-7803.

170. Cleland, J. L.; Kensil, C. R.; Lim, A.; Jacobsen, N. E.; Basa, L.; Spellman, M.; Wheeler, D. A.; Wu, J. Y.; Powell, M. F., Isomerization and formulation stability of the vaccine adjuvant QS-21. *Journal of Pharmaceutical Sciences* **1996,** *85* (1), 22-28.

171. Cabrera, S. G.; Jang, J. H.; Kim, S. T.; Lee, Y. R.; Lee, H. J.; Chung, H. S.; Moon, K. D., EFFECTS OF PROCESSING TIME AND TEMPERATURE ON THE QUALITY COMPONENTS OF CAMPBELL GRAPE JUICE. In *Journal of Food Processing and Preservation*, John Wiley & Sons, Ltd: 2009; Vol. 33, pp 347-360.

172. Yi, J.; Huang, H.; Liu, Y.; Lu, Y.; Fan, Y.; Zhang, Y., Fabrication of curcumin-loaded pea protein-pectin ternary complex for the stabilization and delivery of β-carotene emulsions. *Food Chemistry* **2020,** *313*, 126118.

173. Zhang, R.; Zhang, Z.; Kumosani, T.; Khoja, S.; Abualnaja, K. O.; McClements, D. J., Encapsulation of β-carotene in Nanoemulsion-Based Delivery Systems Formed by Spontaneous Emulsification: Influence of Lipid Composition on Stability and Bioaccessibility. *Food Biophysics* **2016,** *11* (2), 154-164.

174. Gumus, C. E.; Davidov-Pardo, G.; McClements, D. J., Lutein-enriched emulsion-based delivery systems: Impact of Maillard conjugation on physicochemical stability and gastrointestinal fate. *Food hydrocolloids* **2016,** *60*, 38-49.

175. Mao, L.; Xu, D.; Yang, J.; Yuan, F.; Gao, Y.; Zhao, J., Effects of small and large molecule emulsifiers on the characteristics of β-carotene nanoemulsions prepared by high pressure homogenization. *Food Technology and Biotechnology* **2009,** *47* (3), 336-342.

176. Gasa-Falcon, A.; Arranz, E.; Odriozola-Serrano, I.; Martín-Belloso, O.; Giblin, L., Delivery of β-carotene to the in vitro intestinal barrier using nanoemulsions with lecithin or sodium caseinate as

emulsifiers. *LWT* **2020**, 110059.

177. Qian, C.; Decker, E. A.; Xiao, H.; McClements, D. J., Physical and chemical stability of β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food chemistry* **2012,** *132* (3), 1221-1229.

178. Qian, C.; Decker, E. A.; Xiao, H.; McClements, D. J., Impact of lipid nanoparticle physical state on particle aggregation and β-carotene degradation: Potential limitations of solid lipid nanoparticles. *Food Research International* **2013,** *52* (1), 342-349.

179. Li, R.; Tan, Y.; Dai, T.; Zhang, R.; Fu, G.; Wan, Y.; Liu, C.; McClements, D. J., Bioaccessibility and stability of β-carotene encapsulated in plant-based emulsions: impact of emulsifier type and tannic acid. *Food & function* **2019,** *10* (11), 7239-7252.

180. Yuan, Y.; Gao, Y.; Zhao, J.; Mao, L., Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Research International* **2008,** *41* (1), 61-68.

181. Boon, C. S.; McClements, D. J.; Weiss, J.; Decker, E. A., Factors influencing the chemical stability of carotenoids in foods. *Critical reviews in food science and nutrition* **2010,** *50* (6), 515-532.

182. de Carvalho, L. M. J.; Gomes, P. B.; Godoy, R. L. d. O.; Pacheco, S.; do Monte, P. H. F.; de Carvalho, J. L. V.; Nutti, M. R.; Neves, A. C. L.; Vieira, A. C. R. A.; Ramos, S. R. R., Total carotenoid content, α-carotene and β-carotene, of landrace pumpkins (Cucurbita moschata Duch): A preliminary study. *Food Research International* **2012,** *47* (2), 337-340.

183. Tan, Y.; McClements, D. J. In *Effect of Calcium Concentration on Lipid Digestion and beta-carotene Bioaccessibility Using INFOGEST Protocol*, JOURNAL OF THE AMERICAN OIL CHEMISTS SOCIETY, WILEY 111 RIVER ST, HOBOKEN 07030-5774, NJ USA: 2020; pp 29-29.

184. Zhou, H.; Dai, T.; Liu, J.; Tan, Y.; Bai, L.; Rojas, O. J.; McClements, D. J., Chitin nanocrystals reduce lipid digestion and β-carotene bioaccessibility: An in-vitro INFOGEST gastrointestinal study. *Food Hydrocolloids* **2021,** *113*, 106494.

185. Zhang, R.; Zhang, Z.; Zou, L.; Xiao, H.; Zhang, G.; Decker, E. A.; McClements, D. J., Enhancement of carotenoid bioaccessibility from carrots using excipient emulsions: influence of particle size of digestible lipid droplets. *Food & function* **2016,** *7* (1), 93-103.

186. McClements, D. J., Theoretical prediction of emulsion color. *Advances in colloid and interface science* **2002,** *97* (1-3), 63-89.

187. Zheng, B.; Zhang, Z.; Chen, F.; Luo, X.; McClements, D. J., Impact of delivery system type on curcumin stability: Comparison of curcumin degradation in aqueous solutions, emulsions, and hydrogel beads. *Food Hydrocolloids* **2017,** *71*, 187-197.

188. Zheng, B.; Zhang, X.; Lin, H.; McClements, D. J., Loading natural emulsions with nutraceuticals using the pH-driven method: formation & stability of curcumin-loaded soybean oil bodies. *Food & function* **2019,** *10* (9), 5473-5484.

189. Djordjevic, D.; McClements, D.; Decker, E., Oxidative stability of whey protein-stabilized oil-in-water emulsions at pH 3: Potential ω-3 fatty acid delivery systems (Part B). *Journal of Food Science* **2004,** *69* (5), C356-C362.